THE UNIVERSITY *of* EDINBURGH

Title	Cellular functions of Msx1 and Msx2
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Qualification	PhD
Year	2001

Declaration

I declare

a) that this thesis is composed by myself and

b) that the work is my own, except where otherwise stated.

Cecilia Mercedes Oram

March 2001

Abstract

The homeodomain-containing transcription factors Msx1 and Msx2 are involved in the development of many structures in higher organisms; including teeth, skull, limbs, hindbrain and they have essential, yet apparently redundant, functions during mouse eye development. In this thesis I have investigated the practical application of an *in vitro* assay to study Msx cellular functions and Msx downstream genes. I review the literature on eye development and the roles Msx1 and Msx2 may have regulating cell differentiation, signalling, division and death in different developmental contexts. Previous work showed that ectopic expression of *Msx2* in primary cultures of chick pigmented retinal epithelium (PRE) cells promotes a small proportion of transfected cells to develop a neural-like phenotype and to downregulate expression of the key pigmentation transcription factor Mitf. In the experimental work described in this thesis I show that ectopic expression of *Msx2* in dedifferentiated chick PRE cells promotes the formation of cells with a neural-like phenotype. Using dedifferentiated PRE cells substantially increases the number of cells available to study the functions of *Msx1* and *Msx2*. The formation of the small number of neural-like cells in the *Msx2*-transfected PRE cultures is independent of serum growth factors. However, the proportion of *Msx2*-transfected cells developing a neural-like phenotype is not markedly increased by neural-specific culture conditions. I have found no evidence of an increase in PRE cell proliferation as a result of ectopic *Msx2* expression. Interestingly, ectopic expression of *Msx1* in PRE cells also promotes the development of a neural-like phenotype in a small number cells and results in downregulation of Mitf. This suggests that, at least in these cellular functions, *Msx1* and *Msx2* are functionally redundant. To test the *in vivo* relevance of the *in vitro* cellular assay, I analyzed transgenic mice designed to express *Msx2* ectopically in the PRE with βGal and neomycin produced from an internal ribosome entry site (IRES). Mice in one transgenic line showed patches of β Gal reporter gene in the PRE suggesting activity of the *Msx2* transgene in some cells. However, no ectopic *Msx2* expression could be detected by *in situ* hybridization.

Transgenic mice were produced without the IRES βGeo cassette to investigate whether this was negatively affecting *Msx2* transgene activity. Mice from stable transgenic lines and transient transgenic embryos did not show ectopic *Msx2* expression when assayed by *in situ* hybridization. The *in vitro* system provides an assay for the cellular and developmental functions of the Msx proteins and points to a developmental context where these could be investigated *in vivo*.

Acknowledgements

I would like to thank some people without whose wisdom, help and support this thesis would not have been possible. Firstly, I would like to thank my supervisor Duncan Davidson for his inexhaustible patience and enthusiasm and his support throughout this project. Never stop exploring, Duncan.

Ralph Holme for his practical advice and support. In the lab I would like to thank Susan, Lorna, Liz, Allyson, Laura, Aswin, Siobian, Colin, Bob, James and Paul Perry for all their help. I would also like to thank the staff at the Transgenic Unit for their help. For their comments on this thesis I would like to express by thanks to Paula, Colin, Bob and Janet. Thanks to James and Jacob for the lifeline of regular lunchtime banter.

I would like to thank my friends for hatching escape plans, the stellar company and laughter-induced-pain. Made everything bearable.

Finally, I would like to thank my unique and parents for their unfailing support. Mama mia, thank you for the music, for your moral support and outrageous fun. John, thank you for being simply exceptional and for hanging in there with me and giving me the strength to keep going throughout.

Cecilia Oram

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Abbreviations

A	adenine
aa	amino acid
AER	apical ectodermal ridge
A-P	apical-proximal
ATP	adenosine triphosphate
βGal	βGalactosidase
βGeo	βGalactosidase and neomycin
BMP	bone morphogenic protein
BMPR	bone morphogenic protein receptor
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C	cytosine
C-terminal	carboxyl-terminal
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
CNCC	cranial-derived neural crest cells
CNTF	ciliary neurotrophic factor
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DIG	digoxygenin
d	day of incubation
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
dlx	distalless homeobox
DMSO	dimethylsulfide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate

dpc	days post coitum
dpp	depentaplegic
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
EtOH	ethanol
ey	eyeless
FCS	fetal calf serum
Fgf	Fibroblast growth factor
flb	fuzzy little ball
G	guanine
GCL	ganglion cell layer
GFP	green fluorscent protein
gsc	goosecoid
hh	hedgehog
hrs	hours
ihh	indian hedgehog
IGF	insulin growth factor
INL	inner nuclear layer
IRES	internal ribosomal entry site
kb	kilobase
lox	locus of recombination
mins	minutes
MITF	micropthalmia transcription factor
ml	microlitre
mRNA	messenger ribonucleic acid
Msx	muscle segment homeobox

µg	microgram
µl	microlitre
N-terminal	amino-terminal
NaAc	sodium acetate
NR	neural retina
osc	osteocalcin
PAX	paired homeobox
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
Pdgf	platelet derived growth factor
PFA	paraformaldehyde
PRE	pigmented retina epithelium
ptc	patched
r	rhombomere
RA	retinoic acid
rpm	revolutions per minute
RNA	ribonucleic acid
RORβ	retinoic acid orphan receptor β
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
sdH_2O	sterile distilled water
SDS	sodium dodecyl sulphate
SE	surface ectoderm
secs	seconds
sFRP2	secreted frizzled related protein 2
shh	sonic hedgehog
so	sine oculis
Sox	Sry related homeobox
T	thymine

TESPA	3-aminopropyl-triethoxy silane
TF	transcription factor
TGFβ	transforming growth factor β
toy	twin of eyeless
tRNA	transfer ribonucleic acid
Trp	tyrosinase related protein
Tyr	tyrosinase
UV	ultraviolet light
wt	wildtype
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YAC	yeast artificial chromosome
ZPA	zone of polarizing activity

All units are Standard International (SI) units.

Chapter 1 Introduction

1.1 The Msx gene family

The *Msx* genes are a class of homeobox genes which appear to have important functions during the embryonic development of a diverse number of structures. Three *Msx* genes have been cloned in mouse, but recently *MSX4* has been identified in humans (Pollard and Holland, 2000). *Msx1* and *Msx2* were cloned as a result of their homology to *Drosophila* Antennapedia (Hill *et al.*, 1989) and *Msh* (Muscle segment homeobox), (Robert *et al.*, 1989) from which they derive their name. Over the 60 amino acid homeodomain, *Msx1*, *Msx2* and *Msx3* are 92%, 92% and 90% structurally homologous, respectively, to the *Drosophila* gene, *Msh* (Holland, 1991). The aim of this thesis is to investigate the cellular functions of *Msx1* and *Msx2* during the development of the mouse eye. There may be some functional conservation between *Msh* and the vertebrate *Msx* genes.

1.1.1 Expression and function of *Msh*

Msh has roles in the development of subsets of neural and muscle cell precursors. In a *Msh* loss-of-function mutant muscle progenitors form normally, but they fail to recruit surrounding cells and give rise to the appropriate number of founders (Nose *et al.*, 1998). During *Drosophila* neurogenesis *Msh* is expressed in two longitudinal bands, proneural clusters and as development proceeds in individual neuroblasts (D'Alessio and Frasch 1996; Lord *et al.*, 1995). In a *Msh*-null mutant cell division and migration are affected in a subset of dorsal neuroblasts (Isshiki *et al.*, 1997). These results suggest that *Msh* has functions in cell division and signalling in subsets of muscle and neural progenitors. Do vertebrate *Msx* genes have related functions?

1.1.2 Expression of *Msx1*, *Msx2* and *Msx3*

The expression patterns of *Msx1*, *Msx2* and *Msx3* during mouse development have been characterised. The expression patterns of *Msx1* and *Msx2* have been studied at numerous stages of development in *Xenopus*, chick, quail and mouse. In some locations expression of *Msx1* and *Msx2* is conserved in these different organisms, suggesting conservation in the pathways patterning gene expression. *In situ* hybridization has shown that in the gastrula-stage *Xenopus* embryo, *Msx1* is expressed in the ventral ectoderm (Maeda *et al.*, 1997; Suzuki *et al.*, 1997; Yamamoto *et al.*, 2000). In the mouse *Msx1*, expression is seen in the mesoderm and ectoderm, lateral mesoderm and dorsal neuroepithelium (Davidson *et al.*, 1991; Hill *et al.*, 1989; Robert *et al.*, 1989; Takahashi and Le Douarin, 1990). From this early expression pattern, complex, focused patterns of expression appear in diverse organs, (Davidson, 1995) including the developing limb bud, (Hill *et al.*, 1989; Davidson *et al.*, 1991; Robert *et al.*, 1989; Takahashi and Le Douarin, 1990), the prospective neural retina, (Monaghan *et al.*, 1991), the endocardial cushions of the heart (Chan-Thomas *et al.*, 1993; Hill *et al.*, 1989; Robert *et al.*, 1989), the neural roof plate, (Takahashi *et al.*, 1992), the face and head, including the developing follicle and papilla, the cranial neuroectoderm, the forming skull bones, the choroid plexus, Rathke's pouch and the otic vesicle, (MacKenzie *et al.*, 1991a; MacKenzie *et al.*, 1991b; MacKenzie *et al.*, 1992). Cell specific expression of *Msx1* has been observed in cells migrating from the somites, in mice with the *LacZ* reporter gene integrated into the *Msx1* locus (Houzelstein *et al.*, 1999; Houzelstein *et al.*, 2000). *Msx1* and *Msx2* are expressed during the development of a range of different structures which all involve epithelial-mesenchymal interactions. Prompting the questions; what are the cellular functions of *Msx1* and *Msx2* during development and how can they be explored?

A third member of the mouse *Msx* gene family, *Msx3*, was found to be expressed only in the dorsal portion of the neural tube (Wang *et al.*, 1996). This sole domain of expression of *Msx*3 overlaps with both *Msx1* and *Msx2* at early stages. In older embryos, *Msx*3 expression becomes restricted to the ventricular zone of the dorsal neural tube, whereas *Msx1* and *Msx2* become localized to the non-neuronal roof plate

region (Wang *et al.*, 1996). In early development of the neural tube *Msx1*, *Msx2* and *Msx3* may have similar roles in specification of neural progenitors, but their functions in later stages may have diverged. In some of the other locations where *Msx1* and *Msx2* are expressed, for example the eye, they may be involved in neural specification. An interesting route to explain functional redundancy between the vertebrate *Msx* genes is to investigate whether the family originated from duplications of the gene ancestral to *Msh*.

1.1.3 Origin of the *Msx* genes

A single *Msx* gene has been found in the primitive deuterosomes; sea urchin (*Strongylocentrotus purpuratus*), ascidia (*Molgula oculata*) and amphioxus (Holland *et al.*, 1994; Bell *et al.*, 1993; Holland *et al.*, 1994; Ma *et al.*, 1996; Sharman *et al.*, 1999). In these three organisms, *Msx* expression is seen in endodermal and/or mesodermal cells undergoing morphogenetic movements during gastrulation, and in neural and/or ectodermal cells in the anterior portion of the embryo, (Dobias *et al.*, 1997; Ma *et al.*, 1996; Sharman *et al.*, 1999). These expression patterns suggest that similar to *Msh* the *Msx* gene may be involved in regulating cellular specification and differentiation in early vertebrate embryo development. The functions of *Msx1* and *Msx2* in higher vertebrates evolved from the functions of the *Msx* gene in these ancient organisms, but how did the *Msx* gene family arise?

Studies of the chromosomal locations of related homeobox genes, in human and mouse, suggest that the *Msx* genes were part of a homeobox gene cluster which underwent duplications to yield four descendent arrays (Pollard and Holland, 2000). Subsequent to duplication these clusters appear to have been split by chromosome rearrangement (Kume *et al.*, 1998). The duplications potentially included the regulatory regions immediately adjacent to *Msx,* but, via chromosome rearrangements, they may have come under the influence of different long-range regulatory elements. This may have introduced variations in the regulation of expression between the duplicated genes.

In fish many genes have apparently undergone multiple gene duplications. Zebrafish have at least five *Msx* homeobox genes; *MsxA, MsxB, MsxC, MsxD* and *MsxE,* which do not directly correspond to *Msx1*, *Msx2* and *Msx3* in mammals (Ekker *et al.*, 1997). Phylogenetic comparisons of protein sequences indicate that the *Msx* genes from zebrafish are not orthologous to the *Msx1* and *Msx2* genes of mammals, birds and amphibians (Ekker *et al.*, 1997). Zebrafish *MsxB* and *MsxC* are more closely related to each other and to the mouse *Msx3*. The combinatorial expression of the zebrafish *Msx* genes in the embryonic dorsal neuroectoderm, visceral arches, fins and sensory organs suggests functional similarities with the *Msx* genes of other vertebrates, but differences in the expression patterns prevent precise assignment of orthological relationships (Ekker *et al.*, 1997). The number of genes is consistent with current ideas about multiple gene duplications during evolution of fish (for review see;Meyer and Schartl, 1999). It is possible that distinct duplication events gave rise to the *Msx* genes of modern fish and other vertebrate lineages. Investigating the expression and functions of the *Msx* genes in lower organisms may help reveal their functions in higher organisms.

The *Msx* gene family originated from gene duplications, which were then followed by chromosome rearrangements raising some very interesting questions. Which molecular interactions are conserved between the Msx proteins and, following from this, what is the degree of conservation in cellular processes? In the functionally important homeodomain the Msx1, Msx2 and Msx3 proteins show a high degree of conservation suggesting that molecular interactions made by the homeodomain of Msx1, Msx2 and Msx3 may be conserved. The first question is; what do Msx proteins interact with directly via the homeodomain?

1.2 Molecular structure and function of the Msx proteins

Originally the homeodomain was identified as a DNA-binding domain and one of the functions of the Msx homeodomain appears to be binding the regulatory regions of genes. However, several transcription factors have also been shown to mediate interactions with other proteins via their homeodomains. To build up a picture of the molecular functions of the Msx proteins the following section brings together data on

in vitro interactions made by Msx proteins and data on a number of homeodomain mutations associated with human disease.

Fig 1.1 below shows an overview of the Msx protein, the interactions and mutations of the homeodomain are summarized in Fig 1.2.

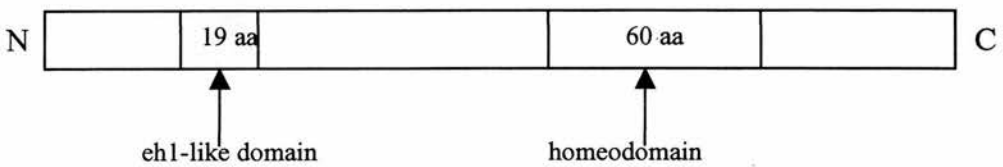


Fig 1.1 Schematic diagram of Msx protein showing the relative positions of the conserved homeodomain and eh1-like domain.

1.2.1 Msx homeodomain-DNA interactions

The 60 amino acid homeodomain differs by only two amino acids between Msx1, Msx2 and Msx3 (Davidson, 1995). Based on sequence comparisons the Msx homeodomain has been classified as a class II or engrailed-like homeodomain (Treisman *et al.*, 1992). Determination of the 3D structure of the engrailed homeodomain has shown that the protein folds to produce a helix-turn-helix structure composed of an extended N-terminal arm and three alpha helices. When interacting with DNA helix1 and helix 2, closest to the N-terminal, are too far away from the DNA to make many contacts. Helix 3, however, which is perpendicular to the first two helices, fits directly into the major groove making extensive contacts with the DNA (Kissinger *et al.*, 1990).

On the basis of *in vitro* gel retardation assays using short (14bp) random oligonucleotides the sequence; CTTAATTG has been proposed as an Msx1 and Msx2 consensus binding sequence (Catron *et al.*, 1993; Catron *et al.*, 1996) . However, these experiments do not take into account the potential influence of regions of DNA flanking or distant from the target sequence, which may have a

significant effect on Msx protein binding *in vivo*. Furthermore, the artificial context of these *in vitro* interactions means they may not be representative of interactions made by the Msx proteins *in vivo*.

An alternative mechanism Msx1 and Msx2 may bind DNA is via co-factor proteins, several of which have been identified *in vitro*. In *in vitro* assays the protein Mint binds both Msx2 and *Osc* DNA (Newberry *et al.*, 1999), but this interaction needs to be confirmed in a physiologically relevant context. Another DNA-binding co-factor, Miz1, was identified by a yeast two-hybrid screen (Wu *et al.*, 1997). Bacterially expressed Miz1 can interact with bacterially expressed Msx2, in an *in vitro* GST pull-down assay (Wu *et al.*, 1997). In addition, to direct binding via the homeodomain, the Msx proteins may be localized to gene regulatory targets by DNA-binding co-factors. With these alternative mechanisms Msx proteins have the capacity to regulate a large range of genes and are not limited to those containing a Msx homeobox binding site in their regulatory region. Following localization, to the regulatory region of a target gene, Msx proteins may repress gene expression by interactions with other proteins.

1.2.2 Msx proteins may regulate gene expression by binding to transcription machinery

Msx proteins appear to function as transcriptional repressors and one way this may be achieved is by direct interactions between the Msx homeodomain and parts of the transcription machinery. Co-transfection of either *Msx1* or *Msx2* expression constructs in cultured cells repressed expression of a reporter gene whose expression was driven from either the above-mentioned consensus homeodomain-binding site or a genomic homeodomain-binding site, from the *Wnt1* enhancer (Catron *et al.*, 1995); (Catron *et al.*, 1996). *In vitro*-interaction and transient-transfection transcription assays suggest that residues in the N-terminal arm of the homeodomain of Msx1 bind the TBP component of the TFIID complex, associated with the transcription machinery (Zhang *et al.*, 1996). However, both the target DNA and cellular context used for these experiments were artificial and the interactions observed may not represent the mechanism of *in vivo* gene repression by Msx1 and Msx2.

Transient co-transfection and gel shift experiments also suggest that *in vitro,* Msx2 can bind the rat *osteocalcin* promoter (Towler *et al.*, 1994), though, again, this interaction has not been demonstrated in an *in vivo* situation. *In vitro* co-transfection experiments, with the *osteocalcin* (*Osc*) promoter as a target and truncated Msx2 proteins, suggest that residues 132-148, upstream and including the N-terminal arm of the homeodomain are required for repression *in vitro* (Newberry *et al.*, 1997). The residues 132-148 appear to be required for an *in vitro* interaction with the TFIIF components of the basal transcription machinery (Newberry *et al.*, 1997). Repression *in vitro* can be mediated by an interaction between the N-terminal arm of the homeodomain and the basal transcription machinery, but this may be only one of several mechanisms by which the Msx proteins repress gene expression *in vivo*.

One alternative mechanism by which transcriptional repression by Msx proteins may be mediated is by an N-terminal repression domain. In transient transfection assays, truncated forms of *Msx1* and *Msx2,* lacking the homeobox and C-terminal portion, maintain transcription repression function (Catron *et al.*, 1996), suggesting that there is a repression domain in the N-terminal of the Msx protein. Furthermore, sequence comparisons have shown that a 19 amino acid repression domain in engrailed, eh1, is conserved in several homeoproteins from both fly and mouse, including *Msx1* and *Msx2* (Smith and Jaynes, 1996), see Fig 1.1. The eh1 domain may be involved in a repression mechanism which involves interaction with DNA-binding proteins. *In vitro* data suggests that the Msx proteins are capable of multiple interactions, but the challenge which remains is the determination of the molecular interactions they make *in vivo* and their cellular significance.

1.2.3 Msx interact with Pax3 Lhx2 and Dlx transcription factors

In vivo the regulation of gene expression by Msx proteins is not limited to direct or indirect interactions with DNA. There is growing evidence that, during development, cross-repressive interactions between homeodomain transcription factor proteins is a common way for these proteins to regulate each others' activity (Briscoe *et al.*, 2000; Dasen and Rosenfeld, 1999; Papin and Smith, 2000). Msx proteins appear to make cross-repressive interactions with Dlx and other transcription factors. During the development of several structures, including the teeth, branchial arches and limb

ectoderm, the expression patterns of *Msx1* and *Msx2* overlap with members of the *Distal-less-related homeobox* genes (*Dlx*) (Weiss *et al.*, 1995). *In vitro* GST-interaction assays and yeast-two hybrid assays suggest that Msx1 and Msx2 can both form dimeric complexes with Dlx2 and Dlx5 *in vitro* and have the potential to dimerize *in vivo* (Zhang *et al.*, 1997). Truncating and substituting amino acids suggests that the dimerization is mediated via the homeodomains of both proteins and residues in the N-terminal arm of the Msx1 homeodomain appear essential for this interaction (Zhang *et al.*, 1997). Gel retardation assays suggest mutual exclusiveness between DNA-binding and dimerization. Furthermore, co-transfection experiments in primary osteoblasts (which express both *Dlx5* and *Msx2*) have shown that an interaction between Msx2 and Dlx5 de-represses Msx-mediated repression of the transfected *osteocalcin* promoter (Newberry *et al.*, 1998). Msx2 repressive activity in calvarial osteoblasts may be regulated by interaction with Dlx5.

Lhx2 also interacts with Msx proteins. During development, expression of *Msx1* and *Lhx2* overlaps in the limb bud (Bendall *et al.*, 1998a). *In vitro* binding assays, using cellular extracts, indicate that *in vitro* Msx1 and Lhx2 can bind each other via their homeodomains. This interaction is incompatible with DNA-binding (Hu *et al.*, 1998). Binding between Msx1 and Lhx2 may regulate the activity of both proteins. However, the *in vitro* context of this interaction is far removed from that *in vivo* and needs to be investigated in a physiologically relevant context and *in vivo*. The high conservation of the homeodomains, between Msx1 and Msx2, makes it possible that Msx2 may also bind to and be regulated by, Lhx2. Interestingly, *Msx2* and *Lhx2* expression domains overlap in the optic vesicle. Furthermore, *Lhx2*$^{-/-}$ mutant mice fail to form an optic cup (Porter *et al.*, 1997), a phenotype similar to that seen in some *Msx1/Msx2* double null mice (Rauchman *et al.*, 1997). These observations, taken together, suggest that *Msx2*, *Msx1* and *Lhx2* may operate in a common pathway during early stages of eye development. Interactions mediated via the Msx homeodomain with other transcription factors may be a general mechanism by which the Msx proteins regulate cellular differentiation. In common with most of the molecular data described in this section these interactions need to be investigated in a physiologically relevant cellular context.

Msx1 is co-expressed with *Pax3* in the muscle precursor cells which migrate to the limb and their interaction and the regulation of *MyoD* has been investigated in cell culture. Immunohistochemistry has shown that the muscle precursors express Pax3 while they migrate and during their differentiation (Bober *et al.*, 1994). The lower level of *Msx1* expression in migrating muscle precursor cells has been visualized *in vivo* using transgenic mice with LacZ inserted into the *Msx1* locus (Houzelstein *et al.*, 1997; Houzelstein *et al.*, 2000). The *Msx1* ^{nlacZ} mice reveal that *Msx1* is expressed in the migrating limb muscle precursors cells and appears to be downregulated when the muscle cells reach the limb and begin to differentiate (Houzelstein *et al.*, 1999). What are the functions of Msx1 and Pax3 in the differentiation of the migrating muscle precursor cells? In transient co-transfection assays in cell culture Pax3 activates and Msx1 represses, *MyoD* regulatory elements (Bendall *et al.*, 1999). Furthermore, ectopic expression of *Pax3, in vivo* in the forelimb and somites of chicken embryos, activates ectopic *MyoD*. Whereas ectopic expression of *Msx1*, inhibits *MyoD* expression and muscle differentiation. In cell extracts *in vitro*, Msx1 can bind Pax3 via its homeodomain and inhibit DNA-binding by Pax3, but this interaction has not been demonstrated directly *in vivo* (Bendall *et al.*, 1999). These results suggest that a cellular function of Msx1 may be to repress myogenic cellular differentiation in the migrating limb muscle precursor cells by neutralizing the activation of *MyoD* by binding Pax3.

The regions of the Msx homeodomain described in this section which bind DNA, the transcription machinery and other transcription factors *in vitro* are summarized in Fig 1.2. Studies of several familial developmental abnormalities have identified two mutations in the Msx homeodomain associated with human diseases. The structures affected by the Msx mutations are the skull and the teeth. Both skull and teeth express *Msx1* and/or *Msx2* during their development, described in the following sections, which involves epithelial/mesenchymal interactions. So, investigating at how these mutations affect development can provide clues to both the molecular and cellular functions of Msx proteins. The mutations associated with human diseases are also indicated in Fig 1.2.

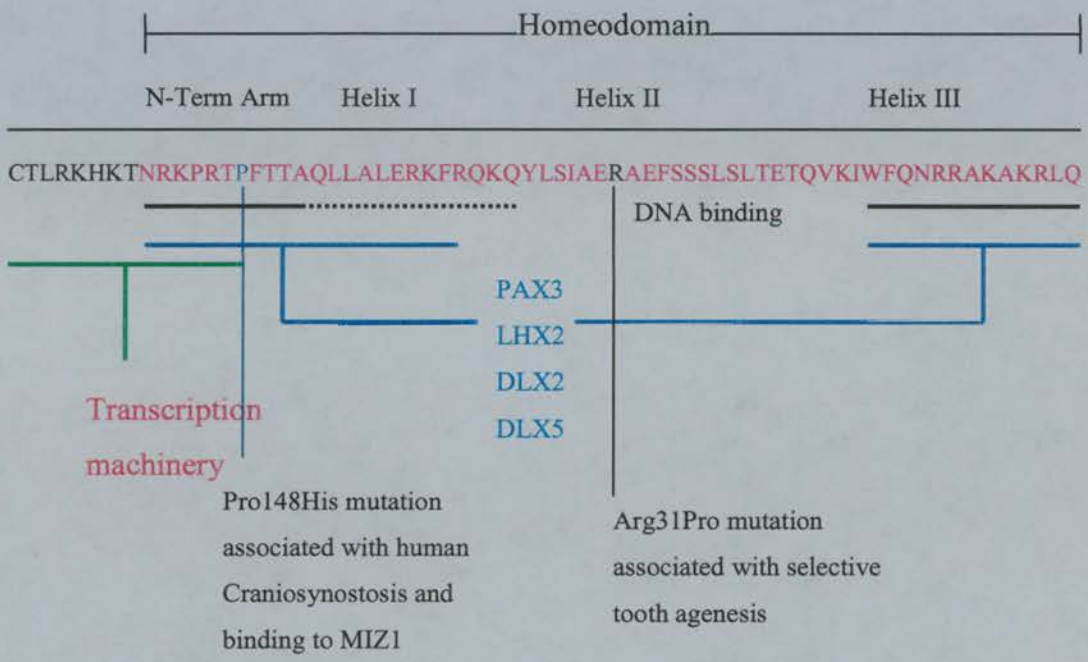


Fig 1.2. Schematic diagram representing the interactions between the Msx homeodomain and flanking regions with protein and DNA. Also included are two *Msx* mutations linked to human diseases. See text for references.

The Pro148His mutation in the MSX2 homeodomain has been linked in humans to Boston Type Craniosynostosis where skull bone fuse prematurely (Jabs *et al.*, 1993). To accommodate the growth of the brain during the late stages of embryonic development the bones of the skull continue to grow. Some of the cells in the sutures, between the bones of the skull, are maintained in a proliferative state. *Msx1* and *Msx2* are expressed in the osteogenic fronts and underlying dura mater (Rice *et al.*,

1998). Abnormal ossification of skull bones is seen in mouse and humans with *Msx1* and *Msx2* loss-of-function mutations (Satokata and Maas, 1994; Satokata *et al.*, 2000; Wilkie *et al.*, 2000). The Pro148His mutation may affect several of the functions of MSX2 since it is in a region of the homeodomain which binds DNA, transcription machinery, other transcription factors and the DNA-binding protein, Miz1. *In vitro* titration experiments suggest Pro148His may act as a dominant gain-of function mutation, increasing the association of MSX2 to MIZ1 and therefore to DNA (Wu *et al.*, 1997). In common with the ancient Msx protein and *Drosophila* Msh an essential function of Msx1 and Msx2 during skull development appears to be regulation of cellular differentiation.

The Arg31Pro mutation has been linked with selective tooth agenesis in humans (Vastardis *et al.*, 1996). The various roles of *Msx1* and *Msx2* in tooth development are described in section 1.3.1, but the molecular interactions affected by the Arg31Pro mutation are not known, the region of the homeodomain where it lies has not been associated with any interactions.

We have seen that Msx proteins have the capacity to bind DNA, DNA-binding proteins, transcription machinery and homeodomain transcription factors including Lhx2, Pax3 and the Dlx proteins. The structural conservation between the Msx proteins suggests that the molecular interactions made by the Msx homeodomain described in this section may be common to all Msx proteins. But, the key question is, are these *in vitro* Msx interactions functionally significant and do they represent interactions which occur *in vivo*? The experiments described so far do not address the question of the cellular roles of the Msx proteins during development. The developmental function of a transcription factor can be defined as the genes whose expression it regulates and the effects of this regulation on the cell. Therefore, to understand the developmental functions of the Msx proteins requires identifying, in a physiologically relevant cellular context, which genes and which pathways they are involved in regulating. Tissue recombination experiments using various model systems including, tooth, hindbrain and limb development have been used to explore the developmental functions of the Msx proteins. The following section describes the

In common with the eye, development of all epidermal organs, including teeth, whiskers, hair follicles and mammary glands, depends on epithelial-mesenchymal interactions. The expression of *Msx1* and *Msx2* correlates with the epidermal-mesenchymal interactions of all these structures. The initial morphological development of all these epidermal organs is similar; the epithelium undergoes a local thickening, followed by a local condensation of the mesenchyme beneath it. The epithelium invaginates into the condensing mesenchyme, until it reaches a characteristic bud structure. In *Msx1*-knockout mice, mesenchyme cells fails to condense around the bud and consequently the teeth arrest at the tooth bud stage (Satokata and Maas, 1994). After this stage, the development of these organs diverges, in order to give rise to specialized organs, with very different morphologies, cell types and functions. Many of the same signals regulate the initial inductive tissue interactions suggesting that at least some of the relationships between the components in the genetic network regulating the development of these structures may be conserved.

A series of tissue recombination experiments in the mouse, revealed that the capacity to direct tooth development starts in the epithelium then shifts to the mesenchyme at E11.5 (Lumsden 1988; Mina and Kollar, 1987). Around E14.5, odontogenesis is directed by a specific group of signaling epithelial cells, known as the enamel knot (Thesleff *et al.*, 1996).

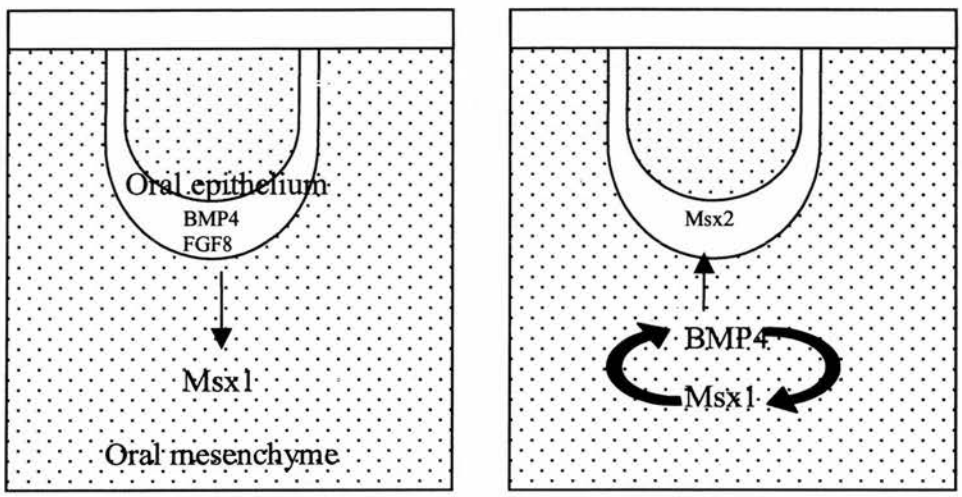
Msx1 may regulate *Bmp4* in the dental mesenchyme. *In situ* expression studies and *in vitro* tissue recombination experiments suggest that early in tooth development, BMP4, FGF8 and FGF9 from the dental epithelium stimulate *Msx1* expression in the dental mesenchyme (Chen *et al.*, 1996; Kettunen and Thesleff, 1998). In the dental mesenchyme *Bmp4* induces its own expression and that of *Msx1*. Furthermore, application of exogenous recombinant human BMP4, to *Msx1*-deficient tooth buds, stimulated them to develop to the early cap stage (Chen *et al.*, 1996). In *Msx1*-deficient dental mesenchyme, *Bmp4* expression is reduced but is preserved in *Msx1*-mutant epithelium (Chen *et al.*, 1996). In *Msx1*-mutant dental mesenchyme, *Bmp4* cannot induce its own expression, suggesting that mesenchymal *Bmp4* expression

may require *Msx1* (Chen *et al.*, 1996). Through the E14.5 cap stage of tooth development, *Msx1* expression is required in the dental mesenchyme for tooth formation (Bei *et al.*, 2000). These results suggest that Msx1 may regulate *Bmp4* in the dental mesenchyme, potentially in a positive feedback loop.

Members of the FGF-family are also involved in *Msx1*-mediated signalling interactions between dental mesenchyme and epithelia. *Fgf3* is expressed in the dental mesenchyme from the bud stage (Thesleff and Vaahtokari, 1992). *Fgf8* expression is preserved in *Msx1*-mutant epithelium, while *Fgf3* is not detected in *Msx1*-mutant dental mesenchyme (Bei and Maas, 1998). Moreover, dental epithelium and recombinant human FGF1-, human FGF2- and mouse FGF8-soaked beads induce *Fgf3* expression in the dental mesenchyme in a *Msx1*-dependent manner (Bei and Maas, 1998). These results suggest that epithelial BMP4 and FGF8 may act in an *Msx1*-dependent fashion to induce expression of members of their respective gene families in the dental mesenchyme.

The relationship between *Msx1* and *Bmp4* in the tooth mesenchyme has also been investigated by various *in vitro* and *in vivo* tissue recombination experiments. *Msx1*-mutant tooth germs were cultured with either recombinant human BMP4 or recombinant FGF3, recombinant human FGF7, human FGF10, human FGF4 or mouse FGF8 for two days *in vitro.* These were then grown under the kidney capsule of syngenic mice to allow complete organogenesis and terminal differentiation (Bei *et al.*, 2000). With this method, *Msx1*-deficient tooth germs, which normally arrest at the bud stage, could be rescued all the way to definitive stages of enamel and dentin formation by addition of BMP4. The transient requirement for *Msx1* expression in the mesenchyme is almost fully supplied by BMP4 alone and not by FGF's. This result strongly suggests that an essential developmental function for *Msx1* in the dental mesenchyme cells is the promotion of *Bmp4* activity, which signals to the dental epithelium at the transition from the bud to the cap stage. *Msx1* and *Bmp4* may form a positive-feedback-loop in the dental mesenchyme, (See Fig 1.3).

Fig 1.3 Summary of the genetic interactions during early tooth development. BMP4 and FGF8 from the oral epithelium stimulate *Msx1* expression in the oral mesenchyme. *Msx1* may form a positive feedback loop with BMP4 in the mesenchyme. The BMP4 from the dental mesenchyme promotes *Msx2* expression in the oral epithelium.



BMP4-signalling from the mesenchyme is important because it induces expression of several key genes in the dental epithelium. Mesenchymal BMP4 has an essential role inducing the epithelial transient signalling centre, the enamel knot, and inducing expression of *Msx2* (Vainio *et al.*, 1993), the cyclin-dependent kinase inhibitor *p21* (Jernvall *et al.*, 1998), *Lef1* (Chen *et al.*, 1996; Kratochwil *et al.*, 1996), *Shh* and BMP2 in the dental epithelium.

These results suggest that Msx1 and Msx2 have functions regulating BMP4 in positive feedback loops during development. The regulation of mesenchymal *Bmp4* could be one of the essential functions of *Msx1* and *Msx2* during vertebrate eye development. *Msx2* is co-expressed with BMP4 in both the surface ectoderm prior to lens placode formation, and in the prospective NR domain of the optic cup. BMP4 may regulate *Msx2* expression in the optic cup and in other tissues. Questions raised by these results are; which genes do the Msx proteins regulate directly to form positive feedback loops with BMP4 and how can this be investigated? The following section describes *in vivo* co-expression studies and cell culture experiments linking *Msx* genes and BMP4 to the cellular process of apoptosis.

1.3.2 In the hindbrain and interdigital mesenchyme *Msx* and BMP4 promote apoptosis

The expression pattern of *Msx2* in the hindbrain coincides with regions undergoing apoptosis. Apoptosis is regulated by extracellular signals, all cells have apoptotic potential and require survival signals to prevent apoptosis. Different cells require different survival signals, but generally, apoptosis requires cell-cell interactions and can be inhibited by cell-matrix interactions. Neural crest cells migrate from the hindbrain to locations around the embryo. Until recently, only even-numbered rhombomeres were thought to produce migrating cranial neural crest cells (CNCC). *In vivo* labelling studies have provided evidence that odd numbered rhombomeres also produce neural crest cells, but that they join streams of cells migrating from adjacent even-numbered rhombomeres (Kulesa and Fraser, 2000). Expression of *Msx2* in the hindbrain, closely correlates with regions undergoing apoptosis, but the role apoptosis plays in elimination of CNCC from odd-numbered rhombomeres remains a controversial issue.

Acridine orange staining of dissected chick hindbrains, showed high levels of apoptosis in rhombomeres 3 (r3) and 5 (r5) (Graham *et al.*, 1993). By *in situ* hybridization, high *Msx2* RNA expression is observed in r3 and r5, and *Msx2* expression preceded apoptosis in a precisely co-localized pattern (Graham *et al.*, 1993). These results suggested *Msx2* may have a role in promoting apoptosis in specific regions of the hindbrain. Isolating or distancing r3 and r5 from their normal position, beside even-numbered rhombomeres, resulted in the down-regulation of *Msx2* and the production of migrating neural crest cells (Graham *et al.*, 1993). One interpretation of these results is that *Msx2*-mediated apoptosis depletes migrating neural crest from odd-numbered rhombomeres. However, removal of rhombomeres from their normal surroundings may remove a negative regulator on neural crest production and a positive regulator of *Msx2* expression.

Whole-mount *in situ* hybridizations on chick hindbrains, show that *Bmp4* is expressed in r3 and r5, and explant co-cultures show *Bmp4* expression depends on neighbouring rhombomeres (Graham *et al.*, 1994). Addition of recombinant BMP4 to

explant cultures of r3 and r5, produced an upregulation of *Msx2* expression and apoptosis (Graham *et al.*, 1994). These results suggest that *Bmp4* may regulate apoptosis in hindbrain via *Msx2*, and also that *Bmp4* expression in odd-numbered rhombomeres may be regulated by even-numbered rhombomeres. Supporting this, *in ovo* adenovirus-mediated ectopic expression of *Msx2* in even-numbered rhombomeres, induced apoptotic elimination of cranial neural crest cells (Takahashi *et al.*, 1998). This suggests that *Msx2*-mediated apoptosis may be one of the mechanisms accounting for the lack of migrating neural crest cells from the odd-numbered rhombomeres, although, the biological significance of neural crest elimination from r3 and r5 is not clear. We can conclude that a cellular function of *Msx2* and *Bmp4* appears to be promotion of apoptosis in certain locations of the hindbrain.

Expression studies and the addition of growth factors *in vivo* implicate the *Msx* proteins and BMP4 in the regulation of apoptosis in a very different developmental situation, in the mesenchyme between the digits. Vital dye uptake, nuclear fragmentation, DNA laddering and TUNEL staining, show the cells between the digits are eliminated by apoptosis (Saunders and Fallon, 1967; Garcia-Martinez *et al.*, 1993; Zakeri *et al.*, 1993, for review see;Hurle *et al.*, 1995). In both chick and mouse *in situ* hybridization shows that *Msx1* and *Msx2* are expressed in the interdigital mesenchyme (Coelho *et al.*, 1991; Davidson *et al.*, 1988; Davidson *et al.*, 1991; Hill *et al.*, 1989; Monaghan *et al.*, 1991). It is reported that *Msx1/Msx2* double null mutants display inhibition of interdigital apoptosis (Chen *et al.*, 1997a). These data suggest *Msx1* and *Msx2* may have an essential function promoting interdigital apoptosis. BMP4 expression in the *Msx1/Msx2* double nulls is reportedly reduced, (Chen *et al.*, 1997a). Furthermore, when *Msx2* was ectopically expressed in the posterior limb mesoderm *in vivo,* by retroviral infection, it reduced cell proliferation, promoted apoptosis and stimulated ectopic expression of *Bmp4* (Ferrari *et al.*, 1998). These results suggest that the role of *Msx1* and *Msx2*'s in interdigital apoptosis may involve regulation of *Bmp4*.

BMP2 and BMP4 have both been implicated in interdigital apoptosis. *Bmp2*, *Bmp4* and *Bmp7* are co-expressed with *Msx1* and *Msx2* in the interdigital mesenchyme (Francis *et al.*, 1994; Helder *et al.*, 1995; Luo *et al.*, 1995; Lyons *et al.*, 1990; Wozney and Capparella, 1993). Blocking BMP-signalling, by expression of a dominant negative *type I BMP* (*dnBMPR-IB*) and *type Ia (dnBMPR-Ia)* receptors, in chicken embryonic hind limbs, greatly reduced interdigital apoptosis, measured by TUNEL staining (Yokouchi *et al.*, 1996; Zou and Niswander, 1996). *BMPR-IB* specifically binds BMP2 and BMP4, and binds BMP7 with low affinity. These results suggest signalling, by these BMPs, may trigger interdigital cell death. *In vitro,* BMP2 and BMP4 can induce apoptosis in mesenchymal cells from the presumptive necrotic zone (Yokouchi *et al.*, 1996). *In vivo* insertion of recombinant human BMP4-soaked beads between the digits of chick limbs accelerated cell death (Ganan *et al.*, 1996). Apoptosis is inhibited by implantation of beads releasing recombinant human TGFβ-1, TGFβ-2, FGF2 and FGF4 (Ganan *et al.*, 1996; Macias *et al.*, 1996). These results suggest that BMP4 expression in the interdigital cells may promote apoptosis, and that TGFβ-1, TGFβ-2, FGF2 and FGF4 may act as survival signals to interdigital cells, which would otherwise undergo apoptosis.

Thus, the study of mutants and tissue recombination experiments has linked the *Msx* genes and BMP4 with the cellular process of apoptosis in certain locations during development. But, how do the *Msx* genes promote apoptosis? Cell-cell interactions promote apoptosis and cell-substrate interactions inhibit apoptosis. Msx proteins may induce apoptosis by blocking cell-cell survival signals. It is difficult to investigate cellular functions *in vivo* because of the inefficiency of artificial gene expression *in vivo* and the contributions of surrounding cells. To explore cellular functions alternative approaches are required, which simplify the cellular situation, but allow the observation in culture to be tested *in vivo*.

Experiments with cells in culture, suggest that cell-cell interactions are required for *Msx2*-induced apoptosis and that this process is inhibited by cell-substrate interactions. P19 embryonal carcinoma cell lines give rise to ectodermal and mesodermal lineages, following aggregation and treatment with retinoic acid.

Ectopic *Msx2* expression in P19 cells results in a two- to five-fold increase in apoptosis induced by aggregation, but has no effect when cells are grown as a monolayer (Marazzi *et al.*, 1997). Addition of BMP4, to P19 cells in monolayers, induces *de novo* expression of *Msx2*, without inducing cell death (Marazzi *et al.*, 1997). Interaction of aggregates with a substrate completely inhibits programmed cell death (Marazzi *et al.*, 1997). These results suggest that cell-cell interactions may be required for *Msx2*-mediated apoptosis and cell-substrate interactions promote cell survival. In the interdigital cells, *Msx2* and *Bmp4* may confer an apoptotic potential, which may require signals from the surrounding cellular environment for apoptosis to be initiated. Alternatively, the *Msx* genes may promote apoptosis by forming a positive feedback loop with BMP4. Another possibility is that the Msx proteins may directly regulate genes that repress the apoptotic pathway? These questions could be investigated by expression of *Msx* genes in cells in culture and analysis of downstream genes and cellular processes. This approach has been used successfully to reveal how the Msx proteins regulate cellular differentiation.

1.3.3 A cellular function of Msx proteins is to stall differentiation

In vitro data suggests that a cellular function of *Msx1* is the stalling of differentiation. *MyoD* is a bHLH transcription factor with a key role in initiating muscle differentiation. In a myoblast cell line and in cultured myotubes, expression of *Msx1* promotes dedifferentiation and down-regulation of expression of the proteins MyoD, myogenin, MRF4 and p21 (Song *et al.*, 1992; Odelberg *et al.*, 2000). Forced expression of *Msx1* in primary human fibroblasts represses *MyoD* enhancer activity and in *in vitro* gel shift assays *Msx1* binds the *MyoD* enhancer (Woloshin *et al.*, 1995). However, the biochemical contexts where these interactions have been demonstrated are very different from those in cells expressing *Msx,* so they may not represent *in vivo* interactions. There is *in vivo* and *in vitro* evidence that Msx1 may inhibit *MyoD* activation by binding the *MyoD* activator Pax3 (Bendall *et al.*, 1999). But evidence for this interaction in a relevant cellular context is required. The Msx proteins may inhibit differentiation by binding directly to the promoters of differentiation genes and/or by blocking the activating action of other homeobox genes.

In vivo Msx1 and *Msx2* are expressed in various populations of proliferating progenitor cells in the skull and limb prior to differentiation. A human mutation in the MSX1 homeodomain has linked it to Boston-Type Craniosynostosis, where the skull bones fuse as a result of premature differentiation (Jabs *et al.*, 1993). The strands of molecular and *in vivo* data suggest the Msx proteins may have roles stalling premature cellular differentiation so a cell culture approach was adopted to investigate this. Multiple mesenchymal and epithelial progenitor cell lines were forced to express *Msx1* and *Msx2* (Hu *et al.*, 2001). Both *Msx1* and *Msx2* were found to stall the differentiation of these different cell lines. The *Msx* genes may connect the pathways regulating the cellular processes of differentiation and proliferation.

Cell cycle genes may be direct regulatory targets of the Msx proteins and this can be investigated in the cell lines forced to express *Msx1* and *Msx2*. Northern and Western analysis from *Msx1*-infected and control-transfected cells shows *Msx1* induces upregulation of *cyclin D1* and Cdk4 kinase activity, but not other cell cycle regulatory genes (Hu *et al.*, 2001). Interestingly, *Msx1* expression does not promote cellular proliferation. *Msx2* also induces an elevation of *cyclin D1*, but the effect is specific to Msx proteins since other *Hox* genes, including the closely related *Dlx* genes do not have the same effect (Hu *et al.*, 2001). *Cyclin D1* expression increased as early as 2 hours after induced *Msx1* expression, so it appears that *cyclin D1* is an early response gene for *Msx1*. But no evidence was found for a direct interaction between *Msx1* and the *cyclin D1* promoter (Hu *et al.*, 2001). Coupled with the evidence that Msx proteins repress transcription is seems likely that *Msx1* upregulates *cyclin D1* indirectly, rather than by direct activation through *cyclin D1* promoter elements. The results of these experiments raise the question whether Msx proteins regulates *cyclin D1 in vivo*. Delayed mammary gland differentiation and elevated *cyclin D1* levels were observed in transgenic mice expressing high levels of *Msx1* in the mammary epithelium (Hu *et al.*, 2001). These results indicate that *in vivo* Msx1 may stall differentiation by elevating *cyclin D1* levels. During development a cellular function of *Msx1*and *Msx2* in progenitor populations may be to maintain *cyclin D1* expression, thereby preventing these cells from exiting the cell cycle and

undergoing terminal differentiation. This may be a cellular function conserved by Msh in Drosophila and the Msx protein in the primitive deuterosomes.

The experiments described to investigate *Msx* developmental functions have included tissue recombination, *in vivo* gene expression studies and analysis of candidate downstream genes and cellular processes in stable *Msx*-inducible cell lines. The evidence from these approaches suggest the *Msx* genes have roles in the BMP4 pathway and regulating the cellular processes of apoptosis and differentiation. The questions which remain to be addressed are; how are the Msx proteins involved in these pathways and processes and which genes are directly regulated by the Msx transcription factors?

1.4 Eye development

We have chosen the eye as the model developmental system to investigate the cellular functions of *Msx1* and *Msx2*. The eye is a popular model system for the study of developmental processes for several reasons. Developmental biology grew from early embryology in the 1890's and for the first half of the 20th century it revolved experimentally around *in vivo* tissue manipulations and the study of naturally occurring mutations. Eyes are clearly visible and easily accessible organs, allowing both the identification of mutant eye phenotypes and experimental manipulation. Subsequently, many of the signals that pattern cell type and genes involved in cell type differentiation in the eye have been identified by *in situ* hybridization, mutants and experiments with cells in culture. To discuss the roles *Msx1* and *Msx2* may have in eye development and provide the background for the cellular assay, explored in this thesis, the following sections describe the molecular basis of vertebrate eye development.

This section introduces several genes which are important for both vertebrate and invertebrate eye development and experiments undertaken to understand the relationships between them. These genes are listed in table 1.1. Many genes involved in *Drosophila* eye formation have been identified and their relationships and functions are being investigated by various *in vivo* and *in vitro* approaches. Genes

involved in vertebrate eye development have been identified either by single gene mutations, resulting in abnormal eye development, or by sequence homology as a result of the apparently conserved genetic network regulating eye development in *Drosophila*.

Some of the relationships between genes may be conserved and investigating their functions in *Drosophila* can help us understand their functions in higher organisms. However, the genetic network regulating vertebrate eye development appears more complex than that of invertebrates. Understanding the regulatory relationships between all these genes and the cellular processes may help us understand the cellular processes and pathways and genes regulated by the Msx proteins during eye development. I will therefore review the role of these genes in eye development and the emerging view of the genetic networks that regulate development of the invertebrate and vertebrate eye.

Table 1.1. Summary of key genes in *Drosophila* eye development and their homologs involved in vertebrate eye development.

Drosophila gene	Vertebrate homolog(s)
Eyeless (*ey*) *Twin of eyeless* (*toy*)	*Pax6*
Dachshund (*dac*)	*Dach*
Eyes absent (*eya*)	*Eya*
Sine oculis (*so*) *Optix*	*Six3* *Optx2*

The transcription factor, Pax6, is critical for vertebrate eye development. Loss-of-function mutations causes *aniridia* in humans and small eye phenotype in mice, where there is an absence or reduction of lens tissue, fusion of the cornea to the lens and in small eye mice a general reduction in the size of the eye (Glaser *et al.*, 1992; Hill *et al.*, 1991). The small eye phenotype in mice was first described by (Roberts, 1967). A dominant mutation affecting embryonic development of the eyes and nose was reported to be responsible for the small eye phenotype (Hogan *et al.*, 1988). In

homozygous small eye embryos, the optic vesicles grow out but there is no lens induction and the nasal pits fail to develop (Hogan *et al.*, 1988). On the basis of comparative mapping studies and phenotypic similarities small eye was suggested to be homologous to congenital *aniridia* (lack of iris) in humans (Glaser *et al.*, 1990; Meer-de Jong *et al.*, 1990). Positional cloning identified a gene, *PAX6*, in the *aniridia* candidate region whose sequence contained a paired-box and a homeobox (Ton *et al.*, 1991). Southern blot deletion analysis and sequencing identified the mutations in *PAX6* responsible for the small eye phenotype (Hill *et al.*, 1991).

During normal eye development of the mouse, zebrafish and chick, *Pax6* is expressed in the surface ectoderm, optic vesicle and lens placode (Krauss *et al.*, 1991; Li *et al.*, 1994; Puschel *et al.*, 1992; Walther and Gruss, 1991). This suggested that *Pax6* may have roles in several tissues at different stages of eye development. Analysis of gene expression in small eye mutant tissue revealed that *Pax6* expression is lost in the surface ectoderm at the time when the lens placode is believed to be specified (Grindley *et al.*, 1995) suggesting that *Pax6* has a function during lens placode specification. In wildtype mice, a broad domain of *Pax6* expression is seen in the head surface ectoderm expression at E8, and later becomes restricted to the lens and nasal placodes. *Pax6* expression is seen in the optic pit at E8, and is maintained in the optic vesicle and cup (Grindley *et al.*, 1995). Initially, *Pax6* is expressed equally in both layers of the optic cup but as retinal development proceeds *Pax6* becomes restricted to the distal margins of the developing retina. *Pax6* expression in the NR becomes restricted to the innermost layer as development proceeds (Grindley *et al.*, 1995). In the developing lens, *Pax6* expression continues in the placode, pit, vesicle and differentiating lens. *Pax6* is also expressed in the developing cornea (Grindley *et al.*, 1995). This expression pattern suggests that *Pax6* may have multiple functions during the different stages in eye formation.

Ectopic expression of *Pax6* induces ectopic eyes in both invertebrates and vertebrates. A number of extraordinary experiments suggested that *Drosophila* could be an extremely valuable tool for understanding eye development and raised interesting questions about eye evolution. The sequence of the *Pax6* gene was found

to be highly conserved between mouse and *Drosophila*, and startlingly ectopic expression of the mouse *Pax6* in *Drosophila* imaginal discs led to formation of ectopic eyes, suggesting conservation of gene function in eye development (Halder *et al.*, 1995; Quiring *et al.*, 1994). These results re-awakened the idea of 'master control' genes (Britten and Davidson, 1969) at the top of a genetic cascade regulating eye development. Several gene families involved in eye development in both *Drosophila* and mouse have been discovered raising intriguing questions about whether eyes in distantly related organisms have a common origin (reviewed in; Gehring and Ikeo, 1999).

Two *Drosophila* homologs of the vertebrate *Pax6* gene, *eyeless, ey*, and *twin of eyeless, toy,* also induce the formation of ectopic eyes when over-expressed in leg and wing imaginal discs (Czerny *et al.*, 1999; Halder *et al.*, 1995). Misexpression of *Pax6* in the head region of *Xenopus* embryos leads to formation of ectopic eyes that contain at least five different mature cell types and have a morphology characteristic of normal eyes (Chow *et al.*, 1999). In vertebrates, *Pax6* is only able to induce ectopic eyes in the head region suggesting that expression of anterior genes or Pax6 co-factors are essential for eye formation and Pax6 function. This may indicate an increasing complexity in the pathways regulating eye development in higher organisms. *Pax6* appears to be able to initiate the genetic programme for eye development and aspects of this programme may be highly conserved between different phyla. *Pax6* may have had an ancient function in primitive light sensitive cells. As these evolved into more complex structures some functions of *Pax6* may have been conserved, and pathway divergence led to recruitment of *Pax6* to additional functions. *Pax6* appears to be just one gene in a complex network regulating eye development in both *Drosophila* and higher vertebrates.

Misexpression of many of the genes in the network regulating eye development in *Drosophila* can generate ectopic eyes. *Eyeless* and *twin of eyeless* are just two of a group of interacting genes essential for *Drosophila* eye development which includes *dachshund* (*dac*), *eyes absent* (*eya*) and *sine oculis* (*so*). All are expressed in the eye primordium and loss of function mutants have severely reduced eyes or no eyes

(Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Mardon *et al.*, 1994; Serikaku and O'Tousa, 1994). Misexpression of these genes leads to ectopic eye formation (Chen *et al.*, 1997b; Pignoni *et al.*, 1997). In *Drosophila*, it has been shown that both *eya* and *so* are regulated by *ey*, but also that *ey* expression is up-regulated by *eya* and *so* (Chen *et al.*, 1997b). To account for this data a self-regulating gene network model has been proposed whose combinatorial activity specifies eye formation (see Fig 1.4, next page), (Chen *et al.*, 1997b). The *Drosophila* gene *optix*, which is related to *so*, induces ectopic eyes in *Drosophila* (Seimiya and Gehring, 2000). A key question is; how conserved are the relationships between genes regulating eye development in invertebrates and vertebrates?

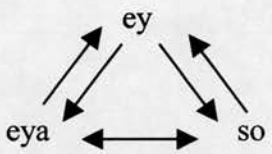


Fig 1.4. Genetic interactions which may occur during *Drosophila* eye development, adapted from Chen *et al.*, 1997.

Vertebrate genes related to *sine oculis* are expressed during eye development and appear to be differentially regulated by *Pax6*. In mouse, 6 genes in the *Six/so* family have been found, however, only the orthologs of *optix*; *Six3* and *Optx2* (*Six6*) are expressed in the eye primordia (Jean *et al.*, 1999; Lopez-Rios *et al.*, 1999; Oliver *et al.*, 1995; Toy *et al.*, 1998; Toy and Sundin, 1999). In the mouse, *Six3* is expressed in the anterior neural plate and the optic vesicle at E9.5; by E11.5, expression is seen in the neural retina, lens and optic stalk (Oliver *et al.*, 1995). At E13.5, expression in the neural retina and lens becomes restricted to the inner neuroblastic layer and anterior lens epithelial respectively (Jean *et al.*, 1999; Oliver *et al.*, 1995). *Six3* expression has also been reported in the PRE (Jean *et al.*, 1999). The effect of a lack of *Pax6* on *Six3* expression in the eye has not been studied, since in small eye homozygous mice, eye formation is almost completely affected (Hogan *et al.*, 1986).

In the brain of the *Pax6*-mutant mice, however, *Six3* expression was unaffected (Oliver *et al.*, 1995). It is possible that in the eye *Six3* could be regulated by *Pax6*.

The gene *Optx*2, which is closely related to *Six3*, shows a similar expression pattern to *Six3* in the eye. Initially, expression of *Optx*2 is seen in the forebrain at E9.5, with expression occurring in the presumptive ventral optic stalk and the ventral portion of the presumptive neural retina (Jean *et al.*, 1999; Lopez-Rios *et al.*, 1999; Toy and Sundin, 1999). In contrast to *Six3*, which is expressed in the entire retinal plate, *Optx*2 is expressed partially in the presumptive ventral neural retina, and no expression of *Optx*2 is observed in the mouse lens. However, expression of chicken *Optx*2 has been reported in the lens placode and lens (Toy *et al.*, 1998) suggesting that there may be interspecies variation in the expression of *Optx*2 and other genes expressed during eye development. These differences may illustrate points where developmental pathways diverged during evolution. From E13.5 dpc in the mouse, *Optx*2 expression is observed in the optic stalk, in the region of the optic chiasm and in the entire neural retina up to E17.5 dpc (Jean *et al.*, 1999). *Optx*2 expression appears normal in the optic vesicles of small eye mice (Jean *et al.*, 1999). This suggests that in the eye, *Optx*2 may not be regulated by *Pax6*. These two orthologs of the *Drosophila* gene, *optix*, may be differentially regulated by *Pax6* during eye development. Their expression patterns suggest that *Six3* and *Optx2* may have roles in multiple processes during vertebrate eye development including specification of retinal cells. Expression of *Six3* and *Optx2* in the eye overlaps with *Msx2* and they may act in the same pathway, either upstream or downstream of *Msx2*.

Mis-expression in vertebrates of the related genes *Six3* and *Optx*2, induces ectopic eye tissue or retinal cell characteristics, implying that they may have functions in the genetic pathway of eye development. Mosaic mis-expression of mouse *Six3* in killifish *O.lattipes* (Oliver *et al.*, 1996) in small clones by injection of plasmid DNA, resulted in the formation of ectopic lenses in the region of the otic vesicle (Oliver *et al.*, 1996). Injection of *Six3* RNA into *lattipes* fish embryos also promotes the formation of ectopic retinal primordia, in the midbrain and prospective cerebellum (Loosli *et al.*, 1999). The ectopic retinal tissue has morphology characteristic of optic

cups and expresses *Rx2,* which is expressed exclusively in the presumptive PRE and NR (Loosli *et al.*, 1999). Ectopic *Six3* expression does not result in formation of complete eyes, suggesting that *Six3* can initiate retinal development, but not fully implement later stages. The effect on mouse eye development of loss-of-function mutations in *Six3* is not known. Injection of *Six3* RNA into *lattipes* embryos causes ectopic *Pax6* expression, and injection of mouse *Six3* RNA initiates ectopic expression of endogenous *lattipes Six3*. These results suggest a *Six3* feedback control loop. Mis-expression studies have revealed that *Six3* appears to have an early function promoting retinal development and may be part of a genetic network regulating eye development, which includes positive feedback mechanisms. *In vivo* expression and mis-expression studies can suggest regulatory targets, but the compensation and complexity of the *in vivo* situation make it difficult to investigate cellular functions with these approaches. An alternative to investigate cellular functions is gene mis-expression in an *in vitro* cellular assay.

In cultured PRE cells, ectopic expression of *Optx*2 induces neural retina markers. In the mouse, *Optx*2 expression is maintained in the neural retina and is not expressed in the PRE, indicating that it may have a function during neural retina development. Ectopic expression of mouse *Optx*2 in primary cultures of E7 chicken PRE, produced upregulation of the neural retina markers; *Chx10* and *visinin,* which are not expressed in the PRE (Toy *et al.*, 1998). The activation of *visinin* appears to be relatively specific to *Optx*2, since it is not seen when mouse *Six3*, *Pax6* or *Eya*2 are ectopically expressed in PRE cells (Toy *et al.*, 1998). Ectopic *Optx*2 can promote the development of neural characteristics in PRE cells, although it is not known whether this represents transdifferentiation of PRE into NR. This suggests that, *in vivo*, *Optx*2 may have a role specifying NR cells, but its expression pattern implies it may also have earlier functions in eye development.

The homologs of *ey* (*Pax6*) and *sine oculis* (*Six3, Optx*2) appear capable of activating the whole or part of the complex interacting gene network which promotes eye development in vertebrates. The approaches described include the study of single gene mutations, knockout mice and *in vivo* expression and mis-expression. The

problem with using these approaches to investigate cellular functions is the complexity of the *in vivo* situation. *In vivo* the network regulating eye development can compensate for a misexpressed gene situation making it difficult to dissect out the cellular roles of the individual components of the network. To understand how all the components of the regulatory network fit together to co-ordinate eye development requires approaches that both simplify the situation and allow simultaneous analysis of a large number of genes. Functional information may come from studying simpler organisms.

If we are going to use simpler organisms, e.g. *Drosophila*, to understand genetic relationships in higher vertebrates it is important to find out; how much conservation is there between the genetic relationships in the regulatory network between *Drosophila* and higher vertebrates. Comparison of the relationships between *ey*, *so* and its closely related gene *optix* and the vertebrate homologs *Pax6* and *Optx2* and *Six3* reveal that some relationships between these genes may be conserved between vertebrates and invertebrates, whereas others are not. In *Drosophila, ey* and *so* regulate each other's expression, however, the relationship between *ey* and *optix* has not been determined. In vertebrates, only the *optix* homologs *Six3* and *Optx2* are expressed in the developing eye. In the vertebrate eye, Pax6 seems to regulate *Six3* in a reciprocal manner, but not *Optx2*. Therefore, it is likely that only some regulatory relationships between genes involved in eye development are conserved between vertebrates and invertebrates.

Interesting new results imply that functions at the cellular level may be conserved between eye genes in *Drosophila* and vertebrates. Functional studies of *hedgehog* (*hh*) and its vertebrate homolog *sonic hedgehog* (*Shh*) suggests that both genes may have a conserved function propagating a wave of neurogenesis during both vertebrate and invertebrate eye development (Heberlein and Moses, 1995; Neumann and Nuesslein-Volhard, 2000). A conserved pathway appears to regulate neurogenesis with homologous genes having similar functions at the cellular and tissue levels. These results have led to the suggestion that vertebrate and invertebrate eyes developed from a more complex organ than previously thought (Jarman, 2000). As

described in this section, the development of vertebrate and invertebrate eyes involves homologous genes but the structures of the two types of eye are very different. An active point of discussion has been whether vertebrate and invertebrate eyes have a common origin or did they evolve independently. The results from the *hedgehog* pathway imply that the common origin of vertebrate and invertebrate eyes was a complex organ and it is not purely coincidental that their development uses homologous genes. Cellular functions may be conserved between genes in *Drosophila* and vertebrates and clues to their cellular functions in vertebrates can be obtained by experiments in invertebrates and *visa versa*.

The vertebrate eye development provides a extensively studied developmental system where the function of the *Msx* genes can be investigated. Many of the key players in vertebrate eye development are common to structures whose development involves epithelial and mesenchymal interactions, in which the functions of the *Msx* genes have been studied. Table 1.2, provides an overview summary of the different stages and processes during vertebrate eye development and some of the genes involved. At the molecular level Msx proteins appear to regulate cellular differentiation, proliferation and death by interactions with DNA and various proteins. The dynamic expression patterns of *Msx1* and *Msx2* during mouse eye development raises the question of whether both genes have roles regulating cellular differentiation, proliferation and death in the lens and retina. *Msx1* and *Msx2* may occupy a key position linking the pathways of differentiation, proliferation and death in the cells of the eye. Determining the cellular function of *Msx1* and *Msx2* may help explain how the eye develops. In the following sections a description of vertebrate eye development is divided into; early inductive interactions, lens development and specification of optic cup neuroepithelium. Many of the genes introduced in the previous section have functions in these different stages of eye development. The key question is; how are *Msx1* and *Msx2* involved in these processes and pathways? To discuss the potential roles of *Msx1* and *Msx2* their expression pattern is described in parallel with the different stages of eye development. In a cell culture approach, to investigate the cellular functions of Msx1 and Msx2 during eye development, could any of the cells expressing them in the eye be cultured *in vitro?*

Table 1.2. Summary of processes at different stages of eye development and some transcription factors and signalling molecules involved. The processes occur in concert and there is crosstalk between them. In addition a few of the transcription factors have roles in several processes.

Process	Transcription factors	Signalling molecules
Early inductive tissue interactions	*Pax6* *sFRP-2* *Sox2*	BMP7 BMP4
Specification of optic vesicle cells as retina	*Rx* *Lhx2*	
Dorso-ventral patterning of the eye	*AHD2* *V2*	RA SHH
Proliferation of retinal neuroepithelial cells	*Chx10*	SHH
Specification of optic cup as NR	*Rx* *Six3* *Otx2* *Pax6*	EGF FGF8
Specification of optic cup as PRE	*Mitf*	activin
PRE differentiation	*Tyrosinase* *Trp1* *Trp2*	
NR differentiation	*Notch* *Hes* *RBP-Jκ/CBF1* *Mash1*	
Lens differentiation	*Pax6* *Sox2* *Prox1*	FGF1 FGF2 IGF1 PDGF

1.5 Expression of Msx1 and Msx2 during vertebrate eye development and 'knockout' mutant phenotypes

The vertebrate eye forms by the co-ordinated development of various tissues, which have very different origins, including the wall of the forebrain; diencephalon, the overlying surface ectoderm and migrating neural crest cells (Fig 1.5 and Fig 1.6). Extensive inductive tissue interactions between these different tissues allows them to develop in concert with each other to produce the complex structure of the eye. During early neuralation (mouse embryonic day 8, E8) the lateral walls of the diencephalon begin to bulge out as optic pits. The optic vesicles form from an evagination of the neural ectoderm and are in contact with the overlying surface ectoderm. At this point, in mouse eye development, *Msx1* is expressed in the perioptic mesenchyme, surrounding the optic vesicle, until E12.5, the last stage analyzed (Holme, 1998). *Msx2* is expressed in the surface ectoderm overlying the optic vesicle and in the distal part of the optic vesicle neuroepithelium; the future neural retina optic vesicle (Monaghan *et al.*, 1991). The lens placode, which is a thickening of the surface ectoderm, is induced by the adjacent optic vesicle neuroepithelium (mouse E9.5).

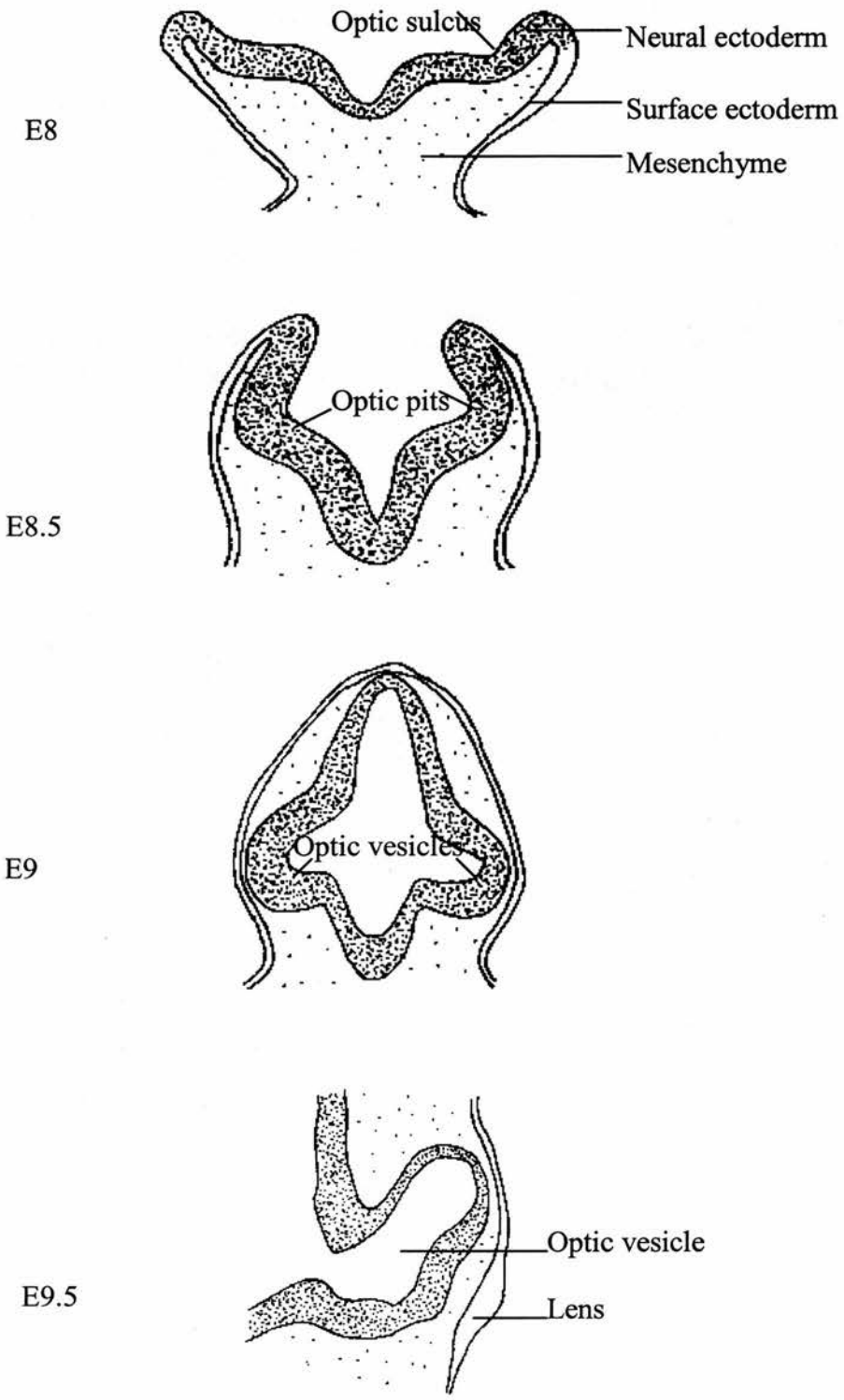
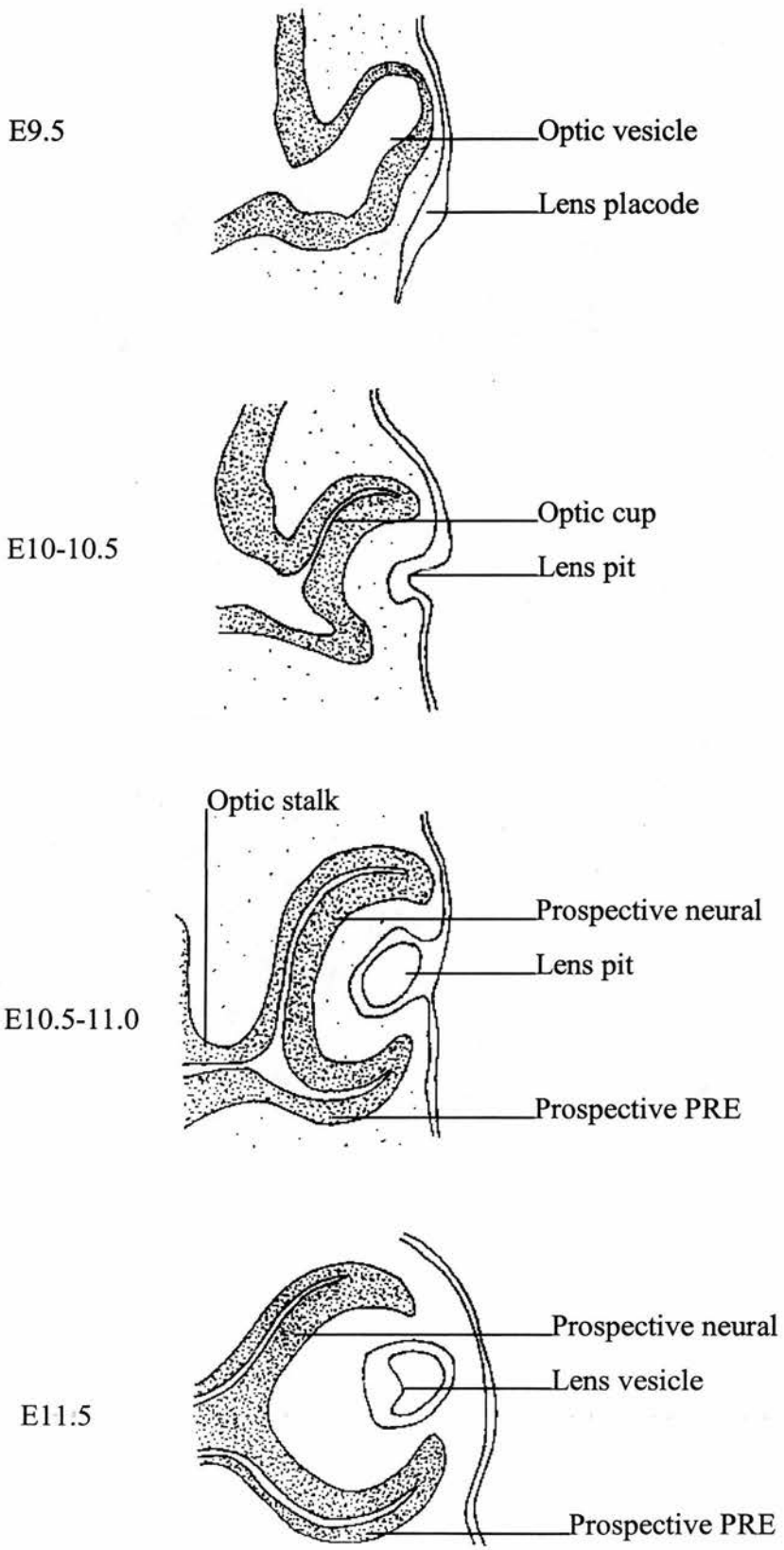


Fig. 1.5 Schematic diagram of early eye development in mouse. The optic sulcus form in the neural ectoderm at E8, giving rise to the optic pits by E8.5. The optic pits evaginate forming the optic vesicles by E9.


E9.5
Optic vesicle
Lens placode
E10-10.5
Optic cup
Lens pit
Optic stalk
E10.5-11.0
Prospective neural
Lens pit
Prospective PRE
E11.5
Prospective neural
Lens vesicle
Prospective PRE

The lens placode develops into the lens vesicle, where *Msx2* is transiently expressed (Monaghan *et al.*, 1991). Signals from the lens induce the optic vesicle to form the optic cup. The expression pattern of *Msx1* and *Msx2* suggests that that they may be involved in the signalling interactions that occur between the surface ectoderm and the neuroepithelium to induce the lens placode and optic cup.

The optic cup neuroepithelium differentiates into a bi-layered structure, forming two very different tissues. The outer layer forms the pigmented retinal epithelium (PRE) and the inner layer the neural retina (NR). In the mouse, from the optic vesicle stage through to formation of the optic cup, *Msx2* is expressed exclusively in the prospective neural retina domain (Holme *et al.*, 2000; Monaghan *et al.*, 1991). Only the dorsal/posterior region of the distal optic vesicle expressed *Msx2* and along the posterior/anterior axis, labelling was detected in approximately the posterior-most 2/3 of the optic vesicle (Holme, 1998). No expression of *Msx2* was observed in the presumptive pigmented retinal epithelium. (Holme, 1998). The distal tip of the optic cup, where the neural and pigmented retinas meet, becomes the iris and the ciliary body. Following optic cup differentiation, *Msx2* expression is restricted to the prospective ciliary body, where its expression overlaps, in a small area, with *Msx1* (Holme *et al.*, 2000; Monaghan *et al.*, 1991). These expression patterns suggest that Msx2 may have a role in patterning the optic neuroepithelium into pigmented retinal epithelium and neural retina.

Intriguingly, mice with null mutations in *Msx1* and *Msx2* indicate, that despite being expressed for the most part in different tissues in the eye, Msx1 and Msx2 appear to have both essential, yet redundant functions during eye development. No eye abnormalities are observed in *Msx1* or *Msx2* single knockout mice (Chen *et al.*, 1997a; Satokata and Maas, 1994). However, *Msx1*/*Msx2* double knockouts are reported to have small or no eyes (Rauchman *et al.*, 1997). The reported phenotypes of the *Msx1*/2 double null mice and *in vivo* expression pattern of *Msx1* and *Msx2* implicate both genes in various processes during eye development including the early inductive tissue interactions, lens development and optic neuroepithelium specification and differentiation.

In the surface ectoderm and optic vesicle of the mouse *Msx1* and *Msx2* are co-expressed with *Pax6* and may lie in the same pathway. As described previously, Pax6 has central roles in the networks regulating both vertebrate and invertebrate eye development. The first stages of mouse eye development is contact between the optic vesicle and surface ectoderm and induction of the lens placode by reciprocal signals between these tissues. Various *in vivo* experiments show Pax6 has functions in both the surface ectoderm and optic vesicle. The purpose of this section is to relate the expression of *Msx1* and *Msx2* to the cellular and molecular processes occurring during the early inductive interactions of eye development, to reveal the functions of these genes.

1.5.1 Early inductive interactions of eye development

Tissue grafting and labelling experiments in *Xenopus* led to a multi-step model for lens induction to be proposed. The model divides the determination of lens ectoderm into four stages; competence, bias, determination and final differentiation (for review see; Grainger 1996). The short period of ectoderm lens competence is apparently regulated by an autonomous developmental timer; when isolated from the embryo and cultured, the ectoderm progresses through a series of competencies (mesodermal, neural and lens) (Servetnick and Grainger, 1991). The nature of the competence remains unknown, but it is during this short period that lens induction commences. Inductive signals from the neural plate and possibly the endoderm and mesoderm, appear to give a large region of head ectoderm lens-forming bias. Head ectoderm from the neural plate stage forms a lens in response to a weak lens inducing environment, but earlier 'competent' ectoderm cannot (Grainger, 1992). The concepts of competence and bias originated before many of the molecules involved in lens development were identified. The progress in defining the molecular basis of the tissue interactions regulating lens development may render the concepts of competence and bias redundant.

At the neural tube stage, the optic vesicles come into contact with the presumptive lens area and seem to have a role during the last stages of lens determination. The optic vesicle may establish the precise location of the lens within the head ectoderm. During later stages of eye development the optic vesicle has a potent effect on lens

cell differentiation. Following lens induction, the induced ectoderm undergoes a series of morphological changes: thickening of the cell layer to form the lens placode, invagination of the placode, formation of the lens vesicle (which is pinched off from the ectodermal layer), and differentiation of the vesicle into lens fibers. Signals between lens placode and neuroepithelium induce the optic vesicle to invaginate and form the optic cup. Signalling interactions from the optic vesicle and cup appear to promote lens differentiation.

The following section describes the molecular and cellular processes occuring in the perioptic mesenchyme, optic vesicle and surface ectoderm during *Msx1* and *Msx2* expression and the inductive interactions of early eye development.

Pax6 is one of the first genes expressed in the eye region and it appears to have essential functions in both surface ectoderm and optic vesicle. Before any signs of eye development, a broad area of *Pax6* expression is seen in the surface ectoderm, at E8.0 in the mouse, which becomes restricted to the future lens placode and optic vesicle (Grindley *et al.*, 1995; Walther and Gruss, 1991). Expression of *Pax6* in the surface ectoderm is maintained in the chick in the absence of the optic vesicle (Li *et al.*, 1994), and in homozygous *Pax6*-null mice activation of *Pax6* in the surface ectoderm is normal but is then subsequently lost (Grindley *et al.*, 1995). These results led to the suggestion that in the surface ectoderm Pax6 may be required for maintenance of its own transcription (Grindley *et al.*, 1995). In homozygous *Pax6*-null mice, initial contact between the optic vesicle and head ectoderm occurs, but there is no evidence of formation of a lens primordium. The optic vesicle subsequently loses contact with the surface and degenerates, which suggests potential defects in surface ectoderm and optic vesicle (Fujiwara *et al.*, 1994; Grindley *et al.*, 1995; Hogan *et al.*, 1986). Tissue recombination experiments, in both rats and mice, show that surface ectoderm from *Pax6*-null mutants cannot respond to signals from a wildtype optic vesicle and the *Pax6*-null mutant optic vesicle cannot induce lens in wildtype surface ectoderm (Enwright and Grainger, 2000; Fujiwara *et al.*, 1994). To further investigate the roles of *Pax6* during eye development chimeric mouse embryos composed of wild-type and *Pax6*-null mutant cells were produced

(Collinson *et al.*, 2000; Quinn *et al.*, 1996). At E9.5 *Pax6*-null mutant cells were found to be eliminated from an area of facial epithelium wider than, but including, the developing lens placode (Collinson *et al.*, 2000). This suggests that *Pax6* has an early function in the broad area of preplacodal head ectoderm. In chimaeras no lens placode formation was seen at a later stage in areas with mutant lens epithelium overlying wildtype optic vesicle cells (Collinson *et al.*, 2000), supporting the idea that *Pax6* has a function in the surface ectoderm essential for lens placode induction.

Pax6 is required for maintaining contact of optic vesicle with lens epithelium and may also have roles in establishing the proximal distal specification of the optic vesicle. In chimaeras segregation of *Pax6*-null mutant and wildtype cells in the optic vesicle occurs at E9.5, probably as a result of different adhesive properties of wild-type and mutant cells (Collinson *et al.*, 2000). In addition, in the presence of a high proportion of *Pax6*-null mutant cells the proximo-distal specification of the optic vesicle, as assayed by the elimination of *Pax6*-null cells distally, is disrupted. Suggesting that *Pax6* has a role in establishment of patterning along the proximo-distal axis of the vesicle (Collinson *et al.*, 2000). Examination of chimaeras with a high proportion of mutant cells indicates that expression of *Pax6* in the optic vesicle is required for maintenance of contact with the overlying lens epithelium. The poor contact made by *Pax6*-null mutant optic vesicles may explain their inefficiency at inducing lens placode (Collinson *et al.*, 2000). Contact between lens epithelium and optic vesicle was found to be preferentially maintained when both are wild-type, however the genotype of the optic vesicle cells is the primary and earlier determinant of adhesion (Collinson *et al.*, 2000). The production of an inductive signal from the optic vesicle to the lens may not depend on *Pax6* directly, but its transduction may require the maintenance of contact between the two tissues.

Pax6 appears to regulate the signalling molecule, retinoic acid (RA), in both optic vesicle and surface ectoderm, during lens induction. *Pax6* influences the ability of the developing head both to produce and respond to RA. Transgenic mice, with a retinoic acid response element fused to LacZ, allow active RA-receptors to be observed qualitatively. RA activity was reduced in small eye mutants, and

application of exogenous RA could not rescue RA-activity in the head surface ectoderm, although it could rescue RA-activity in other regions of the head (Enwright and Grainger, 2000). Co-culturing *Pax6*-mutant and wildtype surface ectoderm and optic vesicles with an RA-sensitive reporter cell line, showed that small eye optic vesicles induce a reduced level of transgene expression, while small eye head ectoderm does not produce detectable levels of RA (Enwright and Grainger, 2000). These results appear to show that *Pax6* affects RA-signalling, in both surface ectoderm and optic vesicle. However, the directness of the effect of *Pax6* on RA and the role of RA in lens induction are not known.

One of the roles *Pax6* may have in the preplacodal surface ectoderm is regulation of expression of *secreted frizzled Related protein*-2 (*sFRP*-2). No *sFRP*-2 expression is detected in homozygous small eye mice, in several locations where the genes are co-expressed, including the prospective lens ectoderm, (Wawersik *et al.*, 1999), suggesting *Pax6* may be involved in the pathway regulating *sFRP*-2 expression.

One signal, which may regulate both *Pax6* and *sFRP*-2 in preplacodal lens ectoderm, is BMP7. Transcripts of *Bmp7* are detected in the optic vesicle and surface ectoderm (Dudley and Robertson, 1997). Expression of *Pax6* in the ectoderm of *Bmp7*-null mice is normal at E8.5, but by E9.5, when the lens placode is forming, no *Pax6* expression is detected (Wawersik *et al.*, 1999). It has been suggested that *Pax6* may regulate its own expression in the head surface ectoderm (Grindley *et al.*, 1995) and BMP7-signalling may be involved in this process. Similar to the down-regulation of *Pax6* in the *Bmp7*-null mice expression, *sFRP*-2 is strongly expressed in the wild-type lens placode but in the *Bmp7*-null mice is not detectable in the ectoderm or optic vesicle, following lens placode formation, from E10.0 onwards (Wawersik *et al.*, 1999). This suggests *Bmp7* has a role maintaining *sFRP*-2 in the pre-placodal lens ectoderm. *Bmp7* may support autoregulation of *Pax6* expression, which in turn, could maintain *sFRP*-2 in the pre-placodal lens ectoderm.

The next stage, in lens placode development, appears to be regulation of *Sox*2 expression. *Sox*2 is expressed in a similar pattern to *sFRP*-2. It is not known whether

expression of *Sox*2 requires *Pax6*, *Bmp7* or *sFRP-2*. No expression of *Sox*2 is detected in small eye or *Bmp7*-mutant mice implying that, in these mice, eye development arrests before *Sox*2 expression (Wawersik *et al.*, 1999). *Sox*2 expression is essential for the later stages of pre-placodal lens ectoderm development.

*Sox*2 expression in the head surface ectoderm is essential for lens development and appears to depend on BMP4. The transcription factor Sox2 has essential functions in the lens placode, regulating expression of *crystallin* genes (Kamachi *et al.*, 1995). No ectodermal expression of *Sox*2 is seen in *Bmp4*-mutant mice (Furuta and Hogan, 1998). Furthermore, *in vivo* application of recombinant human BMP4 rescues ectodermal expression of *Sox*2 in *Bmp4*-mutant mice (Furuta and Hogan, 1998), suggesting that an essential function of BMP4 in the preplacodal surface ectoderm may be direct or indirect regulation of *Sox*2 expression. The relationships these results suggest between *Pax6*, *sFRP-2*, BMP7, *Sox2* and BMP4 are summarized in Fig1.7 below.

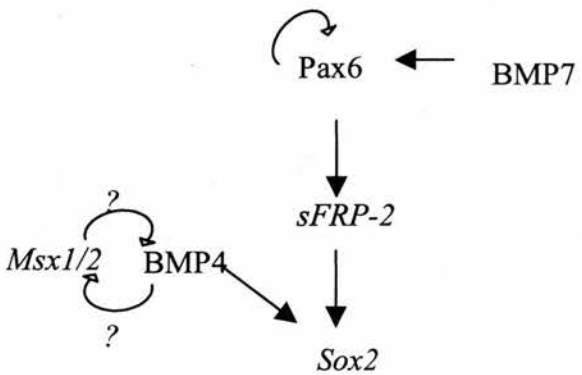


Fig 1.7. Summary of the potential genetic interactions regulating pre-placodal lens formation. *Msx1* and *Msx2* may regulate BMP4 expression.

In this sequence of genetic interactions regulating early eye development *Msx1* and *Msx2* may be involved in regulating BMP4 expression. Analysis of mutants, addition of growth factors and gene expression by i*n situ* suggest that BMP4 may regulate *Msx2* expression in the eye. In the mouse, *Bmp4* expression overlaps with *Msx2*, at E9.5, in both the surface ectoderm and distal optic vesicle (Furuta and Hogan, 1998).

Eye development in *Bmp4*-null mice arrests at the lens placode stage and no expression of *Msx2* is seen in the eye region (Furuta and Hogan, 1998). *In vivo* implantation experiments, of BMP4-carrying beads to *Bmp4*-null mutant eye explants, rescued *Msx2* expression, suggesting that optic vesicle expression of *Msx2* may depend on BMP4. In the mesenchyme surrounding the developing tooth *Msx1* is required to maintain expression of BMP4. The BMP4 from the mesenchyme regulates *Msx2* in the dental epithelium. To explain these relationships it has been proposed that *Msx1* may form a positive feedback loop with BMP4 in the dental mesenchyme. *Msx1* and *Msx2* may also form positive feedback loops with BMP4 in the regulation of apoptosis in the hindbrain and interdigital mesenchyme. *In vivo* *Msx1* and *Msx2* are expressed in various populations of progenitor cells and in progenitor cells in culture they inhibit cellular differentiation. During the inductive tissue interactions of early eye development *Msx1* and *Msx2* may have roles in both the regulation of *Bmp4* and cellular differentiation, potentially inhibiting cellular differentiation via the regulation of BMP4.

The complexity of development *in vivo* and the lack of suitable markers for cellular differentiation make it difficult to investigate the cellular functions of *Msx1* and *Msx2* during this early stage of eye development. The formation of the lens vesicle follows these inductive tissue interactions. *Msx2* is expressed in the lens vesicle and may have a function regulating lens differentiation. Lens development and differentiation has been extensively studied and may provide clues to the cellular function of *Msx2*.

1.5.2 Lens development

The optic vesicle induces thickening of the ectoderm to form the lens placode. This placode invaginates to form the lens vesicle, which is pinched off from the ectodermal layer. In the transformation of the vesicle into lens, the cells in the external side of the vesicle, adjacent to the cornea, form the lens epithelial cell layer maintaining mitotic potential. The cells of the posterior side of the vesicle, facing the retina, differentiate into primary fibre cells (See Fig 1.8). These cells elongate, synthesize crystallin and lose their nucleus. While cells in the center of the

epithelium become quiescent, the cells in the equatorial zone on the margins of the lens epithelium continue to proliferate, and some of these cells differentiate into secondary fibre cells. The transition zone between the lens epithelium and the lens fibers is called the 'bow region', and it is where dynamic changes of cell state take place. The bow region of the lens is exactly opposite the zone where *Msx2* expression in the distal neural retina is seen during optic cup development. So, what are the pathways regulating lens development and do the *Msx1* and *Msx2* genes have roles in these pathways?

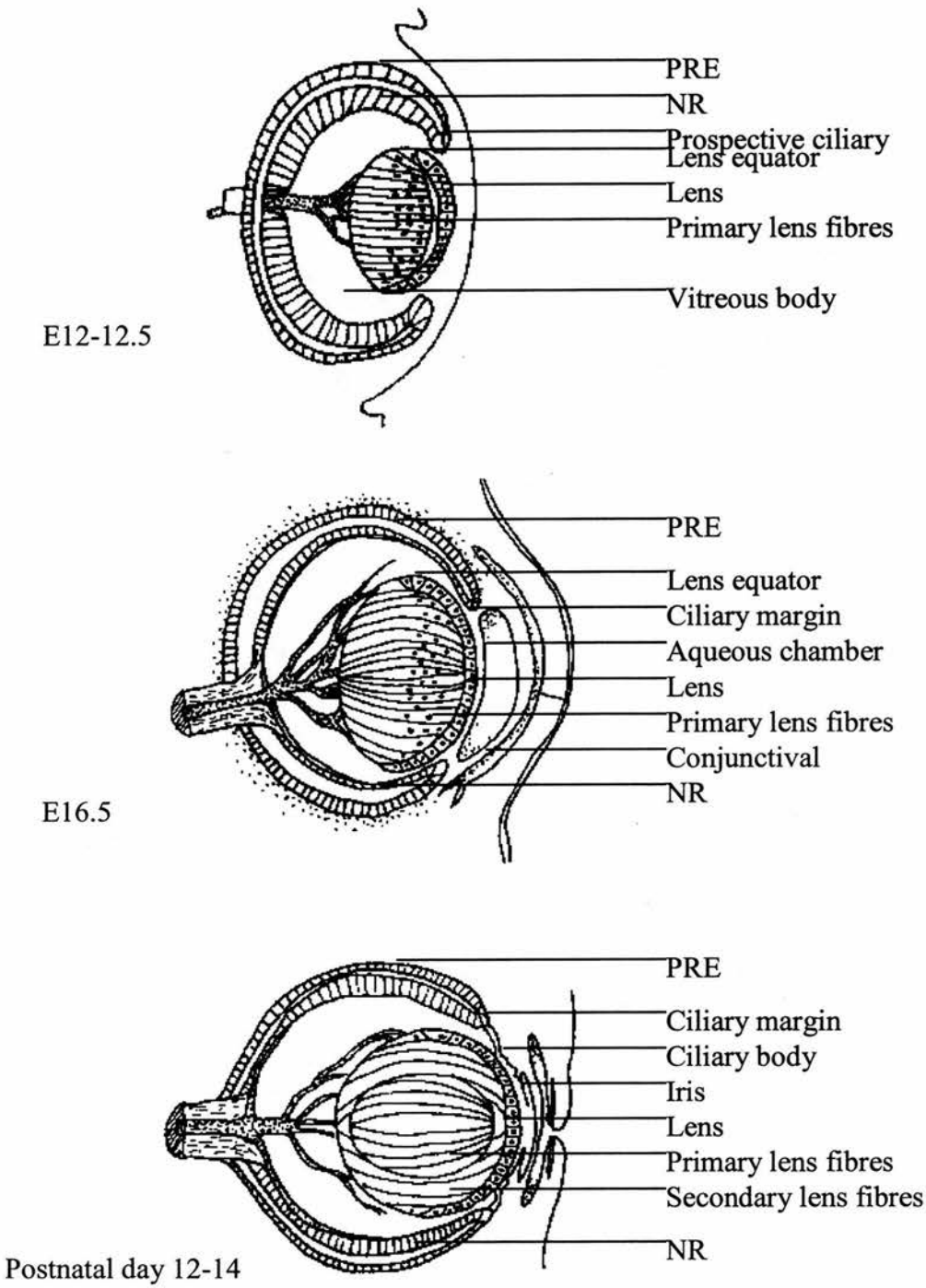


Fig. 1.8 Later stages of mouse eye development.

The differentiation of lens cells is directly regulated by several key transcription factors including; Pax6, Sox2 and Prox1, (Kondoh, 1999). Lens differentiation is regulated by growth factors released into the vitreous humor. Explant experiments and measurement of *in vivo* concentrations show that FGF1 and FGF2 are present in high concentrations in the vitreous humor (Caruelle *et al.*, 1989); (Schulz *et al.*, 1993). In lens explant cultures low concentrations of FGF2 stimulate proliferation and higher concentrations induce differentiation into secondary fibre cells (McAvoy and Chamberlain, 1989). Variation in the concentrations of FGFs in different eye compartments may regulate lens cell differentiation. The retina appears to be a source of extracellular signals regulating lens differentiation. The ciliary body and iris show a high level of immunoreactivity to the FGF1 antibody (de Iongh and McAvoy, 1992) and FGF2 is highly expressed in the NR (de Iongh and McAvoy, 1992). *In situ* hybridization has shown that PDGF is expressed in the iris, ciliary body and IGF1 in neural retina (Reneker and Overbeek, 1996; Delarosa *et al.*, 1994). In cultured chicken lens epithelial cells IGF1 stimulates lens fibre differentiation and *in vivo* over-expression of the *Pdgf-A* isoform in lens epithelium of mice leads to an increase in proliferation and expression of lens differentiation markers (Beebe *et al.*, 1987; Reneker and Overbeek, 1996). *Msx1* and *Msx2* are co-expressed with *Fgf1* and *Pdgf* in the ciliary margin. A potential role of *Msx1* and *Msx2* in the ciliary margin may be the regulation of growth factors that promote lens differentiation. The close proximity of the ciliary margin to the lens equator may facilitate formation of a high local concentration of growth factor in this area. An interesting area to be investigated is how the extracellular growth factors are linked to expression of the transcription factors regulating lens differentiation.

1.5.3 *Msx2* expression in the lens vesicle

Msx2 is expressed in the surface ectoderm in the region from where the lens placode develops and it may have functions both at this early stage of lens development and in later stages. *Msx2* is expressed in the lens vesicle before differentiation. At E10.5 *Msx2* expression is seen in the envaginating lens vesicle (Monaghan *et al.*, 1991). Expression is maintained in the lens vesicle at E11.5, but by E12.5, when the lens vesicle is almost entirely full of fibre cells, no *Msx2* expression is seen (Monaghan *et al.*, 1991). However, *Msx2*-null mice do not display defects in eye development, suggesting that in the lens vesicle *Msx2* may have a redundant function.

Lens development has been studied by tissue recombination experiments, mutant phenotypes, transgenic and chimeric mice, application of growth factors and cell culture. These various approaches have shown that inductive interactions between the lens and retina ensure that both tissues develop in concert. The expression pattern of *Msx2* in the lens vesicle suggest it may have a function regulating lens differentiation. Lens abnormalities are observed in all combinations of *Msx1* and *Msx2* heterozygous and homozygous knockout mice (Maas, unpublished observations). However, a problem in interpreting these results is that lens development involves several tissues that express *Msx1* and *Msx.* This makes it difficult to establish whether the lens defects observed *in vivo* are primary or secondary effects of the lack of *Msx1* and *Msx2*.

The expression patterns and double null mouse phenotype of *Msx1* and *Msx2* suggests both these genes may be involved in the development and differentiation of the optic cup neuroepithelium. The differentiation of retinal cells has been well characterised, making them a good cellular context in which the roles of *Msx2* in cellular processes can be examined, both in a controlled situation in culture and *in vivo*. To understand the potential roles of *Msx2* in the mouse optic neuroepithelium the key questions are; what genes and signals pattern the optic neuroepithelium first into retina and then into PRE and NR? Which genes and signals are involved in regulating cell proliferation and differentiation and how are all these processes co-

ordinated? What maintains cellular differentiation in PRE and NR? Where do *Msx1* and *Msx2* fit into these pathways?

1.5.4 Specification of optic vesicle cells as retina

The neuroectodermal cells at the distal surface of the optic vesicle become specified as retina. *In vivo* expression studies, ectopic expression and mouse null mutants indicate that *Rx* and *Lhx2* are essential for formation of the optic sulci and for optic vesicle invagination, respectively (Mathers *et al.*, 1997a;Mathers *et al.*, 1997b; Porter *et al.*, 1997. An essential early function for *Rx* appears to be assigning anterior and proliferative properties to the rostralmost part of the neural plate. Overexpression of *XRx1* in 8-cell *Xenopus* embryos repressed the anterior neural plate marker *Xotx*2 and also resulted in ectopic pigmented epithelium and overproliferation of the neural retina and neural tube (Mathers *et al.*, 1997b;Andreazzoli *et al.*, 1999). The anterior markers *XPax6*, *Xsix3* and *Xotx*2 were ectopically activated in the hyperproliferative area. This ectopic activation was not seen at the early neural stage and suggests a potential link between proliferation and anterior fate specification (Andreazzoli *et al.*, 1999). *XRx1* loss-of-function mutations result in loss of the anterior structures, telencephalon, ventral diencephalon and eye vesicles, due to early loss of the anterior neural plate territories (Andreazzoli *et al.*, 1999). An early function of *Rx* may be anterior specification of the neural plate, but later functions of *Rx* in the retina remain largely unknown. The possible interactions between *Rx, Six3, Otx2, Pax6, Msx1* and *Msx2* are summarized in Fig 1.9 overleaf.

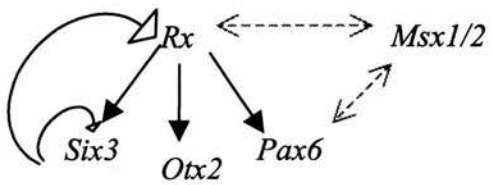


Fig 1.9 Summary of the proposed interactions between the genes *Rx, Six3, Otx2, Pax6, Msx1* and *Msx2* in vertebrates. Black arrows represent downstream regulatory relationships and white arrows feedback relationships.

Mouse mutations, expression studies and *in vitro* culture experiments suggest that Chx10 and Shh regulate proliferation of retinal neuroepithelial cells (Liu *et al.*, 1994; Burmeister *et al.*, 1996; Jensen and Wallace 1997). The expression patterns of *Msx1* and *Msx2* suggest that they are not involved in the specification of the optic vesicle cells as retina, but they may be downstream of these pathways. *Msx2* is expressed in the prospective NR domain of the neuroepithelium suggesting it may have a function regulating NR differentiation. An area which requires further investigation is how the pathways regulating neuroepithelium patterning, proliferation and differentiation relate to each other. These processes may be linked by, for example *Msx1* and *Msx2*.

1.5.5 Specification of optic cup neuroepithelium as pigmented retinal epithelium or neural retina

The optic vesicle invaginates to form the bi-layered optic cup. The outer layer of the optic cup forms the single cell thick pigmented retinal epithelium (PRE) and the inner layer differentiates into the multi-layer neural retina (NR). Specification of these two very different cell types probably involves differential expression of key genes in the prospective PRE and NR domains. It is not known when optic cup specification occurs, but it may begin at the optic vesicle stage, and be associated

with contact of the optic vesicle with the surface ectoderm. In the mouse *Msx2* is expressed exclusively in the prospective NR domain, suggesting it may have a role in regulating NR cell differentiation or suppression of PRE differentiation. Following specification as NR or PRE the two cell types follow well characterised differentiation pathways, providing numerous markers for differentiation.

Upto this point I have mostly used the mouse eye as an example of vertebrate eye development. However, the development *in ovo* of chick embryos makes *in vivo* manipulation and dissection of cells at a particular developmental stage considerably easier than mouse. For this reason chick has been used in many tissue manipulation and primary cell culture experiments, which are described in the subsequent sections. But, it is important to remember that there may be differences in the pathways between chick and mouse.

1.5.6 Differentiation of the neural retina

As a prelude to discussion of the roles of *Msx1* and *Msx2* in the cellular differentiation of the NR and PRE, the following sections describe the molecular basis of NR and PRE differentiation. In contrast to the single cell thick PRE, the mature neural retina is made up of seven different cell types and their cell bodies are arranged in one of three layers (see Fig 1.10). Retinal neurogenesis starts at the centre and proceeds peripherally, with the innermost layers differentiating first and the outer layers last (Young, 1985). The ganglion cells are the first to differentiate and the ganglion cell bodies lie in the ganglion cell layer, which is closest to the lens. A plexiform layer of fibres and synapses separates this layer from the inner nuclear layer, in which the cell bodies of bipolar, horizontal, amacrine and Müller cells are located. The external nuclear layer, the last to differentiate, is adjacent to the pigmented epithelium and contains the cell bodies of the rod and cone photoreceptor cells. The external and inner nuclear layers are also separated by a plexiform layer (Burkitt, Young and Heath, 1993). Differentiation of these seven different NR cell types occurs in a specific order, from a common pool of neuroepithelial precursors. The processes of specification and differentiation are complex and dynamic and appear to be achieved by both changes in the neuroepithelium cells themselves and their environment.

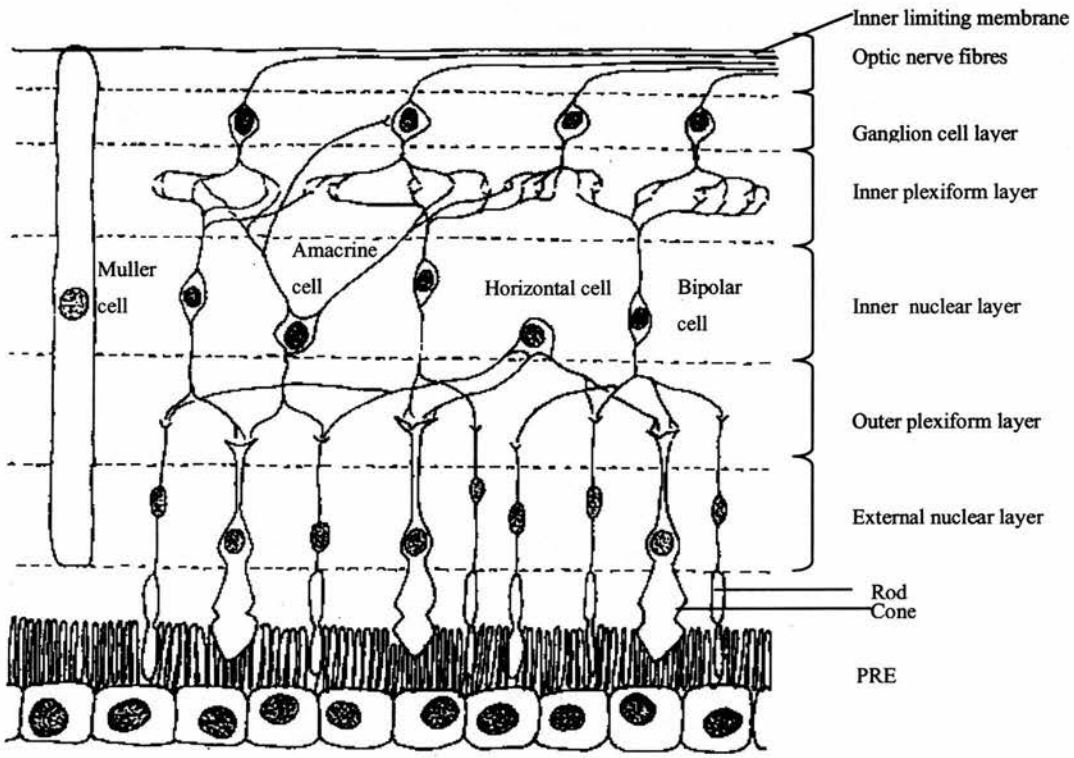


Fig. 1.10 Schematic diagram of the laminar structure of the neural retina. The external nuclear layer is adjacent to the pigmented retina epithelium (PRE) and contains the cell bodies of rod and cone cells. The inner nuclear layer contains the cell bodies of bipolar, amacrine and horizontal cells. Cells of the inner nuclear layer make connections with photoreceptors (rod and cone cells) within the outer plexiform layer. Müller cells stretch between the inner limiting membrane and external nuclear layer. Ganglion cell bodies are located in the inner most layer of the retina, the ganglion cell layer. Diagram adapted from Burkitt, Young and Heath., 1993.

Experiments *in vivo* indicate that retina neuroepithelial cells are multipotent and combined extrinsic cues and intrinsic bias regulate NR cell type (Turner and Cepko, 1987; Alexiades and Cepko, 1997; Ezzeddine *et al.*, 1997; Patel and McFarlane, 2000; Belliveau and Cepko, 1999). Neurogenic selection is mediated by the Notch pathways (for review see; Artavanis-Tsakonas *et al.*, 1995; Lendahl, 1998). In *Xenopus* and several fish species, neurogenesis is not limited to early embryogenesis, and continues at the retinal margin throughout life, with new neurons being generated by a population of stem cells that persist throughout life (Hitchcock and Raymond, 1992; Perron *et al.*, 1998; Raymond and Hitchcock, 1997; Reh and Levine, 1998). Interestingly, using BrdU-labelling a small population of proliferating multipotent retinal progenitors have recently been described in the chick retinal margin (Fischer and Reh, 2000). The results of these experiments appear to indicate that proliferating cells at the retinal margin are able to generate neurons that become incorporated into the GCL and INL and this process may continue in the adult. Could the Msx genes have a role in these multipotent retinal progenitors? *Drosophila Msh* has a function in regulating cell division and signalling in a subset of neural progenitors (D'Alessio and Frasch, 1996; Lord *et al.*, 1995; Isshiki *et al.*, 1997). In both the mouse and chick *Msx1* and *Msx2* are expressed in the retinal margin and may be involved in inhibiting differentiation and maintaining proliferation in the cells in this location (Holme, 1998; Trousse *et al.*, 2001).

1.5.7 Differentiation of the pigmented retinal epithelium

This section describes the genetic basis of PRE differentiation, a process which follows specification and which is important for understanding the functions of *Msx2* in the *in vitro* cellular assay. Mitf is the key transcription factor in PRE differentiation and it regulates several genes in the pigmentation pathway. *In vitro*, co-expression and band shift assays suggest that Mitf may regulate the pigmentation specific genes; *tyrosinase*, *Trp1* and the melanocyte specific gene *QNR71* (Bentley *et al.*, 1994; Turque *et al.*, 1996; Yasumoto *et al.*, 1994; Yasumoto *et al.*, 1997). *Tyrosinase* expression in the outer layer of the optic cup starts at E10.5, before pigmentation, which begins between E11 and E11.5 (Beermann *et al.*, 1992). *Trp1* is expressed in the PRE from E11.5 onwards (Steel *et al.*, 1992). Interestingly, the

pigmentation pathway gene, *Tyrosinase related protein* 2, *Trp*2, does not appear to be regulated by Mitf. *Trp*2 expression is observed in the *Mitf*-null mutant mouse (Nguyen and Arnheiter, 2000). In the mouse, expression of *Trp*2 is first seen at E9.5 in the proximal part of the optic vesicle, which will form the PRE (Steel *et al.*, 1992) and its expression may be promoted directly by signals from the extraocular mesenchyme.

Local reciprocal signals appear to pass between PRE and NR before and during differentiation, which act to promote or maintain their differentiation. Interestingly, in mouse optic vesicle explants where the surface ectoderm has been removed, not only did the distal cells differentiate as PRE but the proximal cells (which normally develop as PRE) differentiated as NR, forming an inverse retina (Nguyen and Arnheiter, 2000). When contact between PRE and NR is prevented, by insertion of a barrier in chick embryos *in vivo*, the outer PRE layer differentiates as NR (Orts-Llorca, 1960). These results suggest signals from the NR may maintain PRE differentiation. Furthermore, targeted ablation of the PRE in mouse embryos, by ectopic expression of diphtheria toxin-A, results in disrupted NR development (Raymond and Jackson, 1995). Where patches of PRE escaped ablation, the laminar structure of the retina was maintained immediately adjacent to these patches (Raymond and Jackson, 1995). Signals from PRE appear to be required for NR development. Mutants, *in vivo* expression studies, explant culture and cell culture experiments have helped identify the signals and genes involved in the patterning specifying, maintaining and differentiation of the NR and PRE domains. The key questions are; how is the optic cup neuroepithelium cells patterned into NR and PRE and does *Msx2* have roles patterning NR or suppressing PRE cell fate?

Study of *Mitf* mutations and ectopic expression in cells in culture provides evidence for the key roles of Mitf in PRE differentiation. In *Mitf*-null mutant mice, the PRE fails to express *tyrosinase* and *Trp1* and expresses neural markers including *Pax6* and *Six3* (Kobayashi *et al.*, 1994; Nakayama *et al.*, 1998; Nguyen and Arnheiter, 2000). Moreover, expression of *Mitf* from retroviral vectors, prevents neural differentiation under *in vitro* conditions where chick PRE cells are otherwise able to

differentiate into a neural cell type (Mochii *et al.*, 1998). Expression of a *Mitf*-carrying retrovirus in chick NR cells, altered their responsiveness to FGF2 and promoted pigmentation (Planque *et al.*, 1999). These results suggest that Mitf activates pigmentation pathway genes, represses neural differentiation genes, and modulates growth factor responses in the developing PRE.

In the mouse signals from the neighbouring tissues may promote *Mitf* down-regulation in the prospective NR cells and pattern the NR and PRE domains of the optic neuroepithelium. *Mitf* expression is first observed in the mouse at E9.0, by *in situ* hybridization, in the whole neuroepithelium prior to invagination (Bora *et al.*, 1998) and by E9.5, *Mitf* expression begins to be restricted to the proximal parts of the optic vesicle, the prospective PRE (Bora *et al.*, 1998; Nakayama *et al.*, 1998). When the surface ectoderm is removed from mouse optic vesicle explant cultures, *Mitf* expression is maintained across the whole neuroepithelium, and the distal-most cells differentiate into PRE, instead of NR (Nguyen and Arnheiter, 2000). This suggests that signals from the surface ectoderm may promote the down-regulation of *Mitf*, in the prospective NR, patterning these cells as NR.

The results of experiments both *in vivo* and in cell culture suggest that FGF growth factors are involved in PRE and NR differentiation. Application of beads, soaked in human recombinant FGF1, FGF2 and murine recombinant EGF, to mouse optic vesicle explant cultures from which the surface ectoderm had been removed, resulted in a down-regulation of *Mitf* expression and maintenance of neural genes, *Pax6* and *Chx10* (Nguyen and Arnheiter, 2000). This suggested that FGF*s* and EGF from the surface ectoderm may promote *Mitf* down-regulation and NR patterning or, alternatively, that FGF*s* and EGF mimic the effects of other signalling molecules from the surface ectoderm. Immunoreactivity shows both FGF1 and FGF2 are expressed in the surface ectoderm, when it is in contact with the optic vesicle. *In situ* hybridization shows that the receptors, *Fgfr1* and *Fgfr2*, are expressed in the optic vesicle (de Iongh and McAvoy, 1993; Pittack *et al.*, 1997; Tcheng *et al.*, 1994; Wanaka *et al.*, 1991). *In vivo* and *in vitro* experiments indicate that both FGF1 and FGF2 are able to promote differentiation of chick PRE into NR. In E4.5 chick eyes,

bovine FGF2-releasing beads appeared to regenerate NR from PRE (Park and Hollenberg, 1989). When supplied with human recombinant and bovine FGF1 and human recombinant FGF2 *in vitro* cultures of aggregated E4.5 chick PRE cells appeared to differentiate into NR cells, (Pittack *et al.*, 1991; Guillemot and Cepko, 1992). These results led to the suggestion that FGF1 or FGF2 from the surface ectoderm may be patterning the NR and PRE domains. The *in vitro* differentiation of NR cells from PRE cells may not be a transdifferentiation, but the result of *Mitf* down-regulation and dedifferentiation by FGF-signalling (Mochii *et al.*, 1988). Mice null for both FGF1 and FGF2 do not have an eye phenotype (Miller *et al.*, 2000), but both genes may be functionally redundant with other genes. The involvement of signals from the surface ectoderm in patterning NR and PRE domains remains unclear, but the differentiation of both these two tissues appears to be influenced by signals from other tissues.

In the chick the signalling molecule, activin, from the extraocular mesenchyme surrounding the eye, is important for promoting and maintaining expression of several PRE genes, including *Mitf.* In cultured chick optic vesicle explants, expression of *Mitf,* the late RPE-specific marker *Wnt13* or melanosomal protein *MMP115* is reduced in most vesicles following removal of the extraocular mesenchyme at both early and late stages of development (Fuhrmann *et al.*, 2000). The NR marker, *Chx10,* is upregulated by removal of extraocular mesenchyme (Fuhrmann *et al.*, 2000). These results suggest signals from the extraocular mesenchyme promote and maintain PRE differentiation, and downregulate NR markers. Addition of activin to the chick optic vesicle explants results in upregulation of the PRE markers *Mitf*, *Wnt13* and *MMP115*, and down-regulation or repression of NR markers *Pax6*, *Chx10* and *Optx*2. Addition of BMP5, BMP7 and GDF5 does not have a comparable effect on gene expression in the explants (Fuhrmann *et al.*, 2000). These results suggest that an activin-signal from the extraocular mesenchyme activates expression of *Mitf*, *Wnt13* and *MMP115.* The extraocular mesenchyme may also down-regulate expression of the neural-retina specific transcription factor genes *Chx10*, *Pax6* and *Optx2*, since in its absence their expression expands throughout the whole optic vesicle. The activin-signal may act

antagonistically with surface ectoderm-derived FGF*s*, which promote NR patterning and inhibit PRE differentiation.

In the chick, *Fgf8* is expressed in the prospective NR, and may have roles in its specification. *In situ* hybridization shows *Fgf8* transcripts are present in the prospective NR domain of the chick optic vesicle (Vogel-Hopker *et al.*, 2000). Interestingly, however, no transcripts of *Fgf8* were detected by *in situ* hybridization in mouse eyes (Lovicu and Overbeek, 1998). Implantation of recombinant mouse FGF8-releasing beads into the mesenchyme surrounding embryonic chick eyes converted the presumptive PRE into NR (Vogel-Hopker *et al.*, 2000). *Mitf* was downregulated, and the NR genes *Rx*, *Sgx1* and *Fgf8* itself were induced, as were the later NR markers; *Cash1*, *islet1*, *synaptotagmin*, *ChAT* and *GABA* (Vogel-Hopker *et al.*, 2000). This suggests that in the chick *Fgf8* may have a role in the pathway that specifies the prospective NR domain. Different genes may be involved in NR specification in mice.

A complex network of interacting genes, including some of the so-called 'master control' genes, appears to be involved in the specification of PRE and NR cells. The following speculative model for some of these interactions and how they may be involved in patterning the NR in the chick can be proposed. This model is summarized in Fig 1.11, however, some interactions suggested may not be conserved between species. In summary, *Fgf8* may be activated in the prospective NR domain by EGF from the SE, upregulating *Rx*. *Rx* is one of the genes activated in chick PRE cells when FGF8 is applied (Vogel-Hopker *et al.*, 2000). In *Xenopus*, Rx upregulates *Pax6* and *Six3* (Andreazzoli *et al.*, 1999; Mathers *et al.*, 1997a). Rx may upregulate *Pax6* and *Six3* in the NR domain. In the prospective PRE domain an activin signal from the mesenchyme may promote and maintain *Mitf* expression. Mitf may then downregulate *Pax6*, *Six3* and *Optx2* and activate *tyrosinase* and *Trp1*, patterning the PRE domain.

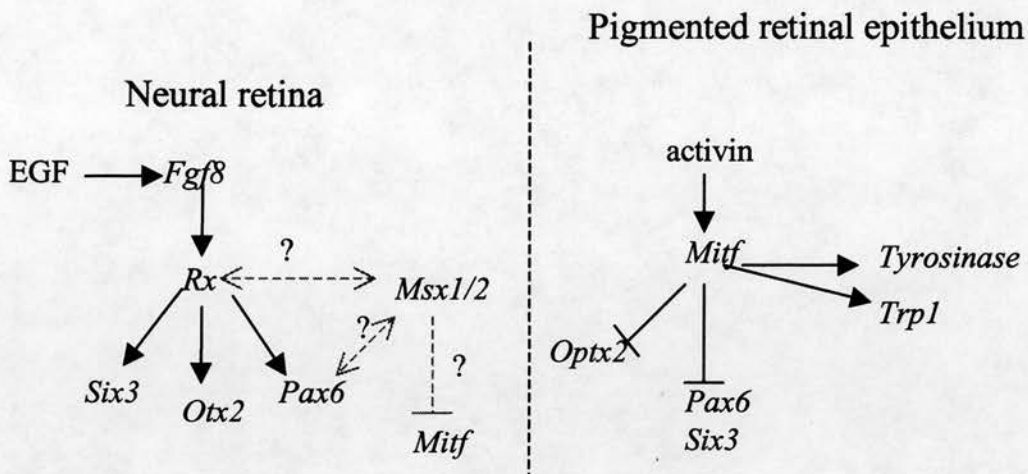


Fig 1.11. Summarizing the model for the genetic interactions, which may occur in the prospective neural retina and pigmented retinal epithelium domains of the chick optic neuroepithelium.

The cells of the optic neuroepithelium are bi-potential. This bipotentiality may be the result of co-expression of transcription factors and their selective repression by local signals from neighbouring tissues, including the ocular mesenchyme. Interference with these signals may tip the balance of the cell in favour of another differentiation pathway.

The PRE and NR share the same common developmental origin, the cells of the optic neuroepithelium. The differentiation of neuroepithelium into PRE and NR has been extensively studied and experiments in culture suggest the two cell types have considerable developmental plasticity. *Msx2* is expressed in the cells of the optic neuroepithelium which will become NR. *Msx2* may have a role patterning NR or suppressing PRE cell fates. In various progenitor cell populations *in vivo* and in cell culture experiments *Msx1* and *Msx2* appear to inhibit differentiation indirectly, via upregulation of *cyclin D1*. Fig 1.12, summarizes the pathways regulating cell specification and differentiation in the pre-placodal surface ectoderm and prospective NR and PRE and the potential roles of *Msx1* and *Msx2*. Do *Msx1* and *Msx2* form positive feedback loops with BMP4 in the surface ectoderm and perioptic mesenchyme? In the pathways that pattern the prospective NR cells, is a function of *Msx2* the downregulation of *Mitf*? PRE cells make a good choice of cell which may respond to *Msx1* or *Msx2* expression and where the answers to these questions can be explored.

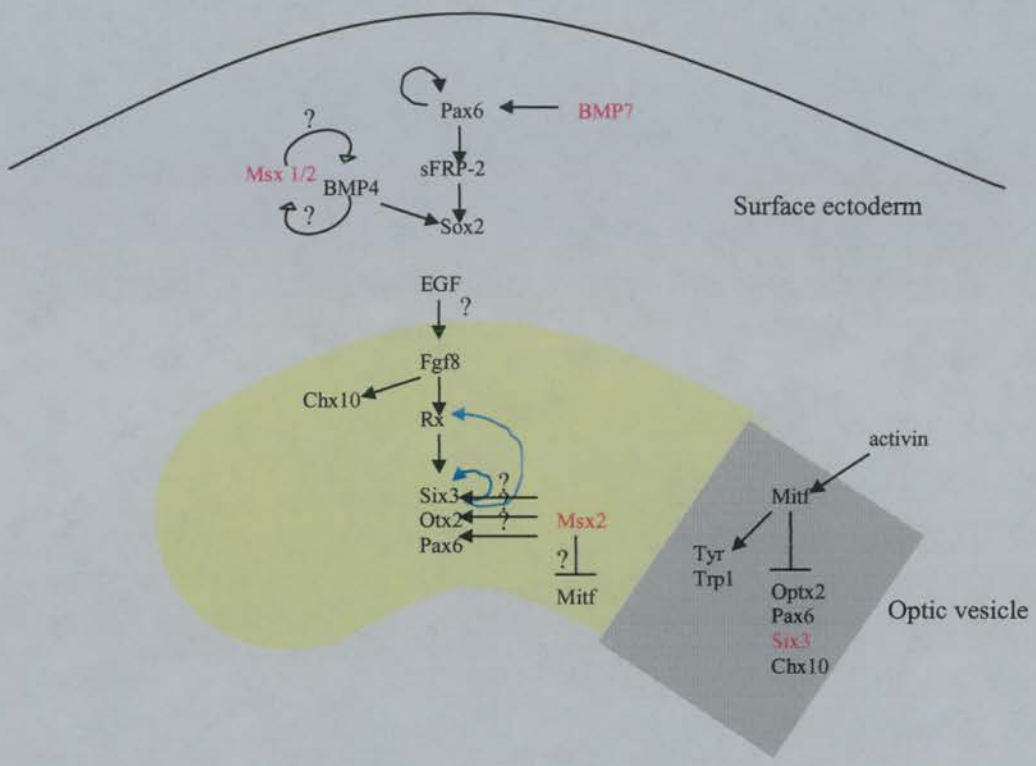


Fig 1.12. Potential genetic interactions in the pre-placodal surface ectoderm, prospective NR (yellow), prospective PRE (grey). Blue arrows represent interactions showed in *Xenopus* relationships or genes in red represent mouse genes.

At later stages of eye development, when the lens and retina are differentiated, *Msx1* and *Msx2* are expressed and in the cells in the ciliary margin, in the transition zone between NR and PRE. *Msx1* and *Msx2* may be involved in inhibiting both PRE and NR differentiation in the ciliary margin, by repression of *Mitf* and other genes.

In the later stages of mouse eye development *Msx1* and *Msx2* are expressed in a small region of the ciliary body. Following optic cup formation at E10.5, *Msx2* expression is seen in the distal half of the optic cup, in the presumptive neural retina, with the proximal boundary of expression located opposite the back of the lens vesicle (Holme, R. H., 1998). This expression pattern is maintained in E11.5 embryos. By E12.5, when the lens vesicle is almost entirely full of fibre cells, *Msx2* is expressed in the presumptive ciliary body, with the proximal boundary of expression located opposite the lens equator (Holme, 1998). Expression of *Msx2* in the distal half of the neural retina is limited to the posterior half and this pattern is maintained at E13.5, the last stage analysed for *Msx2* expression. Following *Msx2* expression in the prospective ciliary body, *Msx1* becomes activated in the ciliary margin, in an overlapping domain. *Msx2* may have a role activating *Msx1* expression in the ciliary margin.

Msx1 expression overlaps with *Msx2* expression in the ciliary margin and extends further round the rim of the optic cup than *Msx2* expression. In the optic neuroepithelium, *Msx1* expression became evident only after E11.5 (Holme *et al.*, 2000). *Msx1* expression was observed in the distal tips of the neural retina, and this expression pattern was maintained through to E19.5 in this region, the presumptive ciliary body (Holme *et al.*, 2000; Monaghan *et al.*, 1991). At E11.5, asymmetrical *Msx1* expression was observed around the rim of the optic cup, with a higher level of on the nasal side of the developing lens (Holme, 1998). In contrast to *Msx2*, which is confined to the dorsal/posterior quarter, *Msx1* was expressed around the dorsal half of the inner layer (Holme, 1998). During the next 24 hours of eye development, *Msx1* expression was activated around the entire distal rim of the neural retina (Holme, 1998). At E12.5 it was possible to distinguish two distinct domains of *Msx1* expression. Within the dorsal half of the neural retina, intense *Msx1* labelling was

detected, while in the ventral half, weaker labelling was detected (Holme, 1998); (Monaghan *et al.*, 1991). A sharp proximal boundary of expression lay opposite the lens equator, with a distal boundary at the junction between the pigmented epithelium and neural retina. By E13.5, the domain of strong *Msx1* labelling had extended further ventrally, so that approximately the dorsal three quarters of the presumptive ciliary margin was now labelled strongly with the *Msx1* probe, while the remaining ventral one third was labelled only weakly (Holme, 1998). The asymmetrical abundance of *Msx1* transcripts is maintained until 16 days p.c. (Monaghan *et al.*, 1991), at this stage, the ciliary body was clearly evident and *Msx1* transcripts were localised in these cells (Monaghan *et al.*, 1991). What are the functions of *Msx1* and *Msx2* in the ciliary body? The cells which express *Msx1* and *Msx2* in the ciliary margin are in the transition zone between PRE and NR. Through the regulation of key genes, for example *Mitf*, *Msx1* and *Msx2* may maintain ciliary margin cells in a proliferative state, inhibiting them from differentiating, illustrated in Fig 1.13.

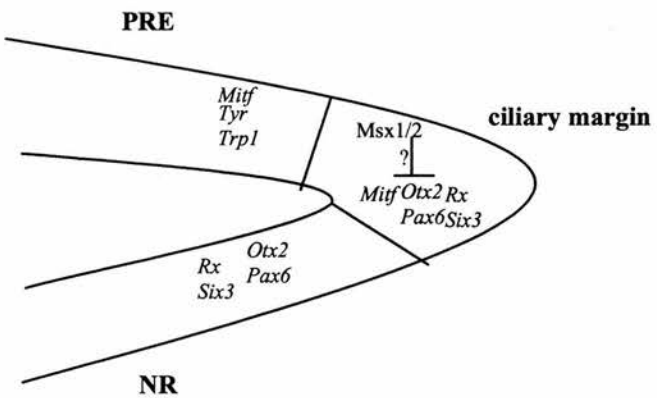


Fig 1.13. *Msx1* and *Msx2* may repress both NR and PRE differentiation in the ciliary margin.

The asymmetric expression pattern of *Msx1* and *Msx2* in the ciliary margin suggests that they may be involved in the dorso-ventral patterning pathways of the eye.

1.5.8 Dorso-ventral patterning of the eye

The *Vax* and T-box gene *Tbx5* and signalling molecules Shh, BMP4 and retinoic acid (RA) have been implicated in dorso-ventral (D-V) patterning of the vertebrate eye. Recent studies indicate that establishment of distinct D-V properties of the developing retina involves the homeobox-containing *Vax* genes (Schulte *et al.*, 1999; Hallonet *et al.*, 1999; Bertuzzi *et al.*, 1999; Barbieri *et al.*, 1999) and the T-box gene *Tbx5* (Koshiba-Takeuchi *et al.*, 2000). In the developing chick optic vesicle, *cVax* and *Tbx5* are expressed in non-overlapping ventral and dorsal domains. The dorsal expression of the *Tbx5* gene appears to be controlled by BMP4, which is normally present in the dorsal optic cup (Koshiba-Takeuchi *et al.*, 2000; Furuta and Hogan, 1998). Misexpression of *Tbx5* results in dorsalization of the ventral retina and, *visa versa*, misexpression of *cVax* causes ventralization, indicated by the loss of ventral and dorsal markers respectively (Koshiba-Takeuchi *et al.*, 2000; Schulte *et al.*, 1999).

BMP4 and Shh appear to act as antagonistic signals patterning the D-V domains of the optic cup. Inhibition of Shh by antibody, in chick optic cups, revealed distinct D-V compartments with different sensitivities to reduction in Shh signals (Zhang and Yang, 2001). Viral-mediated misexpression of Shh in chick differentially altered expression of *Pax6*, *Pax2* and *cVax* in D-V compartments (Zhang and Yang, 2001). Misexpression of Shh abolished the dorsal expression of BMP4 suggesting that ventral Shh may antagonize dorsal BMP4 (Zhang and Yang, 2001). Ventral chick eye morphogenesis and tissue specification was also affected at later stages indicating that the ventral optic cup remain sensitive to Shh levels (Zhang and Yang, 2001). During development of the tooth and limb *Msx1* and *Msx2* have been linked with the regulation of BMP4, potentially in positive feedback loops. The dorsal restriction of *Msx1* and *Msx2* in the ciliary margin matches that of BMP4, suggesting that these genes may lie in the same pathway. What are *Msx1*, *Msx2* and BMP4 doing in the ciliary margin? Recent studies in the chick link expression of *Bmp4, Msx1* and *Msx2* in the prospective dorsal neural retina of the optic cup to spatiotemporally restricted apoptosis (Trousse *et al.*, 2001). So, how are Msx1, Msx2 and BMP4 promoting apoptosis and is the involvement of these genes in the

pathways regulating apoptosis linked to their roles inhibiting differentiation? The genes regulated by Msx1 and Msx2 may have functions in the pathways regulating differentiation, proliferation and apoptosis. The cellular effect of Msx1 or Msx2 expression may be influenced by the cellular context. To find downstream targets of Msx1 and Msx2 the approach we took was to develop a cell culture assay. Msx1 and Msx2 appear to have functions regulating NR and PRE differentiation, making PRE cells a good cell type on which to base the cell culture assay.

In the cell culture assay for *Msx2* function developed in the lab by R. Holme an expression vector, with *Msx2* under control of the Cytomegalovirus (CMV) promoter, is transfected into primary chick PRE cultures. The closest representation of cells *in vivo* are dissected and cultured primary cells. Primary PRE cells from chick, rather than mouse, were used to obtain sufficient numbers of cells. This cell culture assay provides a handle to explore the cellular functions of the Msx proteins and answer the questions; what are the effects of *Msx2* on PRE cell differentiation? Does *Msx2* promote PRE cell dedifferentiation and if so which genes does it regulate directly or indirectly in the process? *Msx2* may promote dedifferentiation by changing cell surface proteins or by directly affecting PRE differentiation genes.

Primary cells in culture do not maintain the full range of contacts with the extracellular matrix and dissociated cells lose many cell-cell contacts. In the absence of the full range of cellular interactions the internal composition of a primary cell in culture may not fully represent cells *in vivo*. In common with mouse, chick PRE does not express *Msx1* or *Msx2* (Holme *et al.*, 2000). Ectopic expression of the mouse protein in chick cells is not ideal, but the amino acid sequence of the homeodomain of mMsx2 and cMsx2 is identical and there is sequence identity in both N and C regions of the homeodomain, suggesting that they have a conserved function (Holme, 1998).

In the cell assay dissociated PRE cells from 5- or 6-day-old chicks are cultured overnight and transfected with a CMV-driven mouse*Msx2* construct. Transfected cells can be identified by expression of β-galactosidase translated from the *IRES*-

β*Geo* cassette. Immunostaining of cultures for both βGal and Msx shows that only 39% of βGal-positive cells were positive for Msx. However, this may be partly due to the relative sensitivities of the immunocytochemistry assays since strong βGal-positive cells were Msx-positive and weak βGal-positive cells were Msx-negative (Holme *et al.*, 2000).

To investigate the effects of mMsx2 on PRE cell differentiation, the expression of the transcription factor Mitf was analysed in the cell culture assay. Immunostaining of cultures of 5-day-old chick PRE cells for both Mitf and βGal showed that only 19% of *mMsx2*-transfected cells were Mitf-positive, compared to 71% of control-transfected cells, (Holme *et al.*, 2000). Seventy-eight percent of untransfected cells, immediately adjacent to *mMsx2*-expressing cells, were Mitf-positive. Ectopic expression of *mMsx2* in cultured PRE cells leads to down-regulation of Mitf in a cell-autonomous fashion (Holme *et al.*, 2000). These results suggest that ectopic *Msx2* expression promotes the down-regulation of the key PRE differentiation protein, Mitf, and therefore promotes PRE cell dedifferentiation. This result raises several questions, for example; how direct is the effect of Msx2 on Mitf? Msx2 may directly downregulate expression of *Mitf* or it may promote PRE cell dedifferentiation, indirectly, via another pathway, which leads to *Mitf* down-regulation. One way Msx1 may repress muscle cell differentiation is by direct binding and regulation of the promoter of the key muscle differentiation bHLH transcription factor *MyoD*. In the pigmentation differentiation programme the bHLH transcription factor Mitf plays an analogous role to that of MyoD in the muscle differentiation programme. Alternatively, Msx2 may bind to and interfere with a *Mitf* transcription activator. Another possibility is that, Msx2 may induce dedifferentiation by upregulating *cyclin D1* and maintaining the PRE cells in a proliferative state. *Msx2* may link the regulation of differentiation and proliferation. Does Msx2 effect PRE cell proliferation? In mouse, *Msx2* is co-expressed with Mitf in the prospective NR domain of the optic vesicle neuroepithelium. The repression of Mitf observed in culture may represent an *in vivo* cellular function of Msx2, but this requires confirmation in the *in vivo* context. Does ectopic expression of *Msx2* in PRE cells in transgenic mice downregulate Mitf?

In addition to the downregulation of Mitf, ectopic *mMsx2* expression in chick PRE cells was found to have a dramatic effect on the morphology of a small number of cells. Six-day-old chick PRE were cultured overnight and transfected with the *mMsx2* expression construct; 48-72hrs later *Msx2*-transfected cells generally had a more irregular shape compared to control transfected cells. Eleven percent of βGal-positive PRE cells in *mMsx2*-transfected cultures have processes more than 10 times longer-than-wide, compared to only 3% of βGal-positive PRE cells in the cultures transfected with the control construct. Ectopic *mMsx2* expression in PRE cells leads to an increase in the proportion of transfected cells with long processes (Holme *et al.*, 2000). A small subpopulation (1-5%) of *mMsx2*-transfected PRE cells displayed a distinct, dendritic morphology, with more than one long process extending from a clearly defined cell body. Cells with this distinctive morphology were never observed in control-transfected primary cultures. Untransfected cells, in the *mMsx2*-transfected culture, did not show a dendritic morphology or extended processes, suggesting that the formation of the dendritic morphology is a cell autonomous effect (Holme *et al.*, 2000). The maximum cellular response, judged by the proportion of transfected PRE cells with a dendritic morphology, was 48hrs after transient transfection with the *mMsx2* construct. The proportion decreased after 72hrs and no cells with a dendritic morphology were observed after 96hrs (Holme *et al.*, 2000). Morphologically, two types of dendritic cells could be distinguished; those with two long processes, generally extending in opposite directions from the cell body, referred to as 'bipolar', and those with more than two processes, which were often highly branched. These results suggest that a cellular effect of ectopic *Msx2* expression is the promotion of neural-cell characteristics in some PRE cells. Raising interesting questions; what are the characteristics of the transformed cells and how directly is expression of m*Msx2* involved in this cellular transformation?

To investigate whether *mMsx2* may be promoting neurogenesis in the PRE cells, expression of several neural markers was analysed in PRE cells, after transient transfection with either the *mMsx2* or control construct. Ganglion cells are the first neural retinal cell-type to differentiate. To test whether the dendritic cells were

ganglion cells, antibodies to the neural markers Gap 43, islet-1, NF68, Map-2 were applied to m*Msx2*-transfected cells, but failed to label them (Holme, 1998). The lack of expression of many neural markers may indicate that the dendritic cells are only partially differentiated neural cells. The *TuJ1*-antibody reacts with class III β-tubulin and has been used as an early neuronal cell type marker (Lee *et al.*, 1990). In 3.5- and 5- day-old chick retina *TuJ1*-immunoreactivity is associated with ganglion cells (Lee *et al.*, 1990). Additional neural retina cell types become *TuJ1*-positive as differentiation proceeds (Holme *et al.*, 2000). *In vivo* at E5, PRE cells are negative for *TuJ1*, however, in tissue removed from the eye, a small population of cells becomes positive for *TuJ1* after less than 17 hrs in culture. Human PRE cells also express *TuJ1* in culture, but not *in vivo* (Vinores *et al.*, 1995).

In primary cultures of PRE cells, from 5- or 6-day-old chicks cultured overnight and then transfected with either control or *Msx2* expression constructs and examined 48 h later, 21% of cells transfected with the control construct were *TuJ1*-positive compared to 43% of the *mMsx2*-transfected cells. Since not all the βGal-positive cells express Msx protein at detectable levels, this experiment was repeated, assaying for class III β-tubulin and Msx immunocytochemically. In cultures transfected with the *mMsx2* construct, 56 ± 13.5% of Msx-positive cells were *TuJ1*-positive, (Holme *et al.*, 2000). In cultures transfected with the control construct, 18 ± 8% of transfected (βGal-positive) cells, were *TuJ1*-positive (Holme *et al.*, 2000). Ectopic expression of *mMsx2* in cultured PRE cells leads to an increase in the number of cells expressing the neuronal marker *TuJ1* (Holme *et al.*, 2000). These results suggest that ectopic *mMsx2* is directly or indirectly promoting the expression of class III β-tubulin, a characteristic of neural cells.

Interestingly, all 30 βGal-positive dendritic cells observed with a bipolar morphology were *TuJ1*-positive (Holme *et al.*, 2000). No bipolar cells were *TuJ1*-negative. In contrast, some multi-polar-transfected cells were *TuJ1*-positive and some were negative (Holme *et al.*, 2000), which may indicate differentiation into different cell types. In the embryonic chick, *TuJ1* immunoreactivity is associated with ganglion cells (Lee *et al.*, 1990). It has been reported that *TuJ1* does not label glial cells

(Trimmer and McCarthy, 1986). The *Msx2*-induced dendritic *TuJ1*-negative cells may be Müller cells (Lee *et al.*, 1990; Trimmer and McCarthy, 1986). It is not known whether these potentially different cell types come from the same PRE cell or from different PRE cells. Ectopic m*Msx2* expression may promote PRE cell dedifferentiation and formation of multipotent progenitor cells, or it may directly drive the differentiation of different neural cell types. Further characterisation is required to determine how *mMsx2* is acting at the cellular level to induce neural cellular characteristics in PRE cells. The small numbers of cells exhibiting these changes is a severe limitation on further investigations, but ectopic *Msx2* expression may be having a cellular effect on a larger proportion of PRE cells than those developing a dendritic phenotype. Indeed, the downregulation of Mitf is a cellular effect seen in a large proportion of cells, thus increasing the basis of the assay for Msx2 cellular function. The formation of dendritic cells raises the questions; how is ectopic *Msx2* expression in PRE cells in culture promoting the development of neural characteristics? Can *Msx2* promote neural characteristics in dedifferentiated cells increasing the number of cells that can be studied.

The differentiation of PRE cells and the effects of cell-cell and cell-ECM contacts have been investigated by growing PRE cells in culture at different densities and using antibodies to block cell-ECM contacts, (Itoh and Eguchi, 1986; Grisanti and Guidry, 1995). These approaches show that cell-cell contacts, between PRE cells, and cell-ECM contacts maintain PRE cell differentiation. They also indicate that physical or chemical disruption of the contacts promotes their dedifferentiation, dissociated PRE cells in culture will therefore dedifferentiate. So, what are the signals passing between neighboring PRE cells which maintain their differentiation?

Addition of FGF2 to PRE cells in culture shows it promotes cell dedifferentiation (Opas and Dziak, 1994; Pittack *et al.*, 1991). This may occur by FGF2-induced changes in cell surface proteins, or for example, by Mitf downregulation. The *in vivo* roles of FGF1 and FGF2 and their relationship to the pathways patterning NR and PRE remain unclear, but they may lie upstream of *Msx1* and *Msx2*.

Cell culture experiments have shown that dedifferentiated PRE cells form a multipotent state, which can differentiate into lens-like cells, or redifferentiate into PRE cells, depending upon culture conditions (Agata *et al.*, 1993; Itoh and Eguchi, 1986). Northern blots have shown that dedifferentiated PRE cells, which redifferentiate into lens cells, pass through a bipotent intermediate cell state. This state is characterised by lack of expression of PRE- or lens-specific genes and increased expression of c-myc (Agata *et al.*, 1993). *Msx2* may promote the formation of multipotent dedifferentiated PRE cells, which may have the capability to redifferentiate into lens or NR cell types.

The results from the cell culture assay suggest that Msx2 may repress *Mitf* the prospective NR domain of the optic vesicle and in the PRE/NR boundary of the ciliary margin. The potential contributions of Msx2 to the pathways regulating specification and differentiation of NR and PRE cells are shown in Fig 1.12. The cellular assay provides a controlled but physiologically relevant system in which to explore the cellular functions of Msx1 and Msx2. In some cells, the downregulation of *Mitf* by Msx2 and promotion of PRE cell dedifferentiation may be sufficient to send some cells down a neural differentiation pathway. In response to growth factors in the media a small number of the *Msx2*-expressing PRE cells may develop neural characteristics. Raising the following questions; are serum growth factors required for the *Msx2*-transfected cells to develop neural characteristics? The lack of neural-specific substrate and media may limit the number of *Msx2*-expressing PRE cells able to develop the neural phenotype. How are culture conditions or growth factors influencing the formation of the cells with neural characteristics? The small number of *Msx2*-transfected cells acquiring the neural-like phenotype is a limitation to the use of the cellular assay to further investigate Msx function and find Msx downstream genes. Therefore, an initial priority was to understand the factors affecting the formation of the dendritic cells and this understanding may increase the number of cells showing an *Msx2*-induced change. In the thesis I examine the effect of serum-growth factors and neural-specific culture conditions on formation of the neural-like phenotype. I also investigate increasing the number of cells by using dedifferentiated PRE cells. The effect on Mitf provided both a Msx2 downstream

gene and greatly increased the number of cells available to assay. The structural conservation between Msx1 and Msx2 makes exploration of conserved cellular functions with the assay an interesting question.

In vivo and in cells in culture the inhibition of differentiation by *Msx1* and *Msx2* has been associated with the maintenance of a proliferative state. *Msx1* and *Msx2* may connect the pathways regulating cell differentiation and division. I use the cellular assay to investigate whether *Msx2* may regulate both PRE cell differentiation and proliferation.

Since ectopic expression of *Msx2* has effects on PRE cells in culture this provides an *in vivo* cellular context to investigate Msx cellular functions. Any cellular effect observed in culture must be confirmed *in vivo* and this is the aim of the transgenic approach taken in the thesis. In summary, the cellular effects of Msx1 and Msx2 in cultured PRE cells and *in vivo* are explored with the aim of using the assay to understand the cellular and developmental functions of these genes during vertebrate eye development.

Chapter 2 Materials and Methods

2.1 Bacterial Cell Culture and Plasmid DNA Preparation

2.1.1 Media and solutions

All chemicals were supplied by BDH unless stated otherwise. Media and solutions were prepared as described in (Sambrook, Fritsch and Maniatis, 1989) and sterilized by autoclaving.

L-broth: 10g tryptone, 5g yeast extract, 10g NaCl, 2.46g $MgSO_4$ dissolved in 1 litre of water.
L-agar: L-broth: 10g tryptone, 5g yeast extract, 10g NaCl, 2.46g $MgSO_4$ and 15g agar (Oxoid Ltd) dissolved in 1 litre of water.

Ampicillin: (Boehringer Mannheim) Stock solution made at 50mg/ml in dH_2O filter sterilised and stored at -20°C. Added to autoclaved media to give a final concentration of 50μg/ml.

2.1.2 Growing bacterial cells on agar plates

A small volume (200μl) of suspended bacterial cells was pippetted onto the surface of the L-agar and spread evenly with a sterile bent glass rod. For bacterial cells from a growing colony or glycerol stock, cells were streaked out onto L-agar plates using a sterile loop. To select for ampicillin resistant colonies, ampicillin was added to the L-agar prior to pouring the plates. The plates were then inverted and incubated for 12-16hrs at 37°.

2.1.3 Preparation of plasmid DNA

Small scale plasmid DNA preparation; a single colony was used to inoculate 6mls of L-broth containing ampicillin and grown in a shaking incubator at 37°C for 12-16 hrs. The cells were then harvested by centrifugation and plasmid DNA isolated using the QIAprep Spin Plasmid Kit (Qiagen)

Large scale plasmid DNA preparation; a single colony was used to inoculate 20mls of L-broth containing ampicillin and grown for 7hrs at 37°C in a shaking incubator. The entire culture was then used to inoculate a further 200mls of L-broth containing ampicillin and grown for 12-16 hrs at 37° in a shaking incubator. The cells were then harvested by centrifugation at 4000rpm for 20mins at 4°C and plasmid DNA isolated using a Plasmid Maxi Kit (Qiagen) as per manufacturers instructions.

2.2 DNA Cloning into Plasmid Vectors

2.2.1 Strain of bacteria used

XL1-Blue MRF' genotype: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lac I^{q}ZΔM15 Tn5 (Kan^{r})]

2.2.2 Preparation of competent cells

Using a sterile loop, bacterial cells from a frozen stock were streaked out onto a L-agar plate and grown at 37°C for 12-16 hrs. A single colony was then used to inoculate 10mls of L-broth which was grown at 37°C in a shaking incubator for 12-16hrs. The entire culture was then used to inoculate 500mls of L-broth and grown at 37°C in a shaking incubator until the culture had an absorbance of 0.7 at 590nm. The cells were then harvested by centrifugation for 5mins at 5000rpm at 4°C. The pelleted cells were washed in 250mls of ice cold 0.1M $MgCl_2$ and re-centrifuged as before. The cells were then resuspended in 250mls of ice cold 0.1M $CaCl_2$ and then incubated on ice for 20mins. The cells were pelleted as before, resuspended in 42.5mls of 0.1M $CaCl_2$ and 7.5mls of glycerol (filter sterilised), aliquots of 300µl were snap frozen in Cryotubes (Nunc) using liquid nitrogen and stored at -70°C.

2.2.3 Plasmid vectors

pSK: pBluescript II SK (Stratagene).

pCI: Mammalian expression vector (Promega).

2.2.4 CMV-driven *Msx1*, *Msx2* and control expression constructs

See Appendix 1 for construct maps.

Msx1

Full-length mouse *Msx1* cDNA was cloned downstream of a CMV promoter and upstream of an IRES-βGeo cassette (gift from Dr. A Smith). This construct also contained a β-globin intron and SV40 polyadenylation signal downstream of the IRES-βGeo cassette.

Msx2

Full-length mouse Msx2 cDNA was digested with *BsmI* (1263) to remove unnecessary 3' untranslated sequence and a phosphorylated adapter, containing a *MluI* site, was ligated. This modified cDNA was cloned into the Promega pCI mammalian expression vector and an IRES-βGeo cassette was inserted downstream.

Control

The IRES-βGeo cassette was cloned into an empty pCI vector.

2.2.5 Electro-transformation of competent cells

Before transformation the DNA solution was desalted by adding the solution to a milipore filter floating on a Petri dish of deionized H_2O and leaving for 45 mins at RT.

An aliquot of competent cells was thawed on ice, and 1µl of the transforming DNA was added, mixed with the cells and left on ice for 1 min. The mixture was then transferred to an ice cold cuvette and pulsed with 2.47kV in a BioRad Gene Pulser. 1 ml of LB was added to the cells and they were transferred to an eppendorf tube and incubated at 37°C for 1 hr, to enable the cells to start to express the ampicillin resistance gene on the transforming plasmid. Following this incubation aliquots of several different volumes were spread onto L-Amp (15g bacti-agar per litre of LB and 20 µg/ml ampicillin) plates and incubated overnight at 37°C.

2.3 Enzymatic manipulation of DNA

2.3.1 Solutions

TE: 10mM Tris, 1mM EDTA (pH 7.5).

2.3.2 Quantification of DNA

The concentration of double-stranded DNA was determined by measuring the absorbance at 260nm in a spectrophotometer. An absorbance reading of 1 corresponds to 50μg of DNA per ml.

2.3.3 Restriction enzyme digestion of DNA

Digestions of DNA with restriction endonucleases were carried out in the appropriate buffer at the recommended temperature. Restriction enzymes were supplied by Boehringer Mannheim and New England Bio Labs. Up to 1μg of DNA was digested in 10-20μl using 1-2 units of enzyme. The reaction was then incubated at the appropriate temperature for 90 mins. When necessary the reaction was terminated by heating at 68°C or 80°C for 15 mins, depending on the heat sensitivity of the enzyme. Double-digests, in which both enzymes can operate in the same buffer, the digests were carried out simultaneously. Otherwise after digestion with one enzyme, the sample was ethanol precipitated between reactions

2.3.4 Dephosphorylation of 5' termini

Calf intestinal phosphatase (CIP) was used to dephosphorylate the 5' ends of the vector molecules before cloning. This prevents recircularisation of vector molecules during the ligation step. DNA was dephosphorylated with 1 unit of CIP (Boehringer Mannheim) in 50μl of 1x CIP buffer (50mM Tris-HCl, 0.1mM EDTA, pH 8.5) at 37°C for 30mins. An additional unit of CIP was then added and the reaction continued for 45mins at 45°C. The reaction was terminated by adding a 1/10 volume of 200mM EGTA (Sigma) and heating to 65°C for 10mins.

2.3.5 DNA ligation

Insert and vector DNA were mixed at a ratio of 3:1 insert:vector, using 10ng of vector DNA. The DNA was ligated by 1 unit of T4 DNA ligase (Boehringer Mannheim) using a Rapid Ligation Kit (Boehringer Mannheim). The DNA was diluted in DNA dilution buffer, as according to manufacturers instructions and added to in 10µl of 1x ligation buffer. After thorough mixing the solution was incubated at RT for 5 mins and spread onto L-Amp plates and incubated overnight at 37°C.

2.4 DNA Electrophoresis

2.4.1 Solutions

20xTBE: 1M Tris.HCl, pH8.0; 20mM EDTA; 1M boric acid, pH8.3.
20xTAE: 0.8M Tris.HCl, pH 8.0; 20mM EDTA; 0.4M acetic acid.
10x DNA Loading Buffer: 20% Ficoll w/v (Pharmacia), 100mM EDTA, orange G (Sigma).

2.4.2 Agarose-gel electrophoresis

DNA molecules were separated according to their size on horizontal agarose gels. Flowgen agarose was used routinely. 0.8% to 2% agarose gels were used depending on the size of the fragments being analysed. All gels were made with 1xTBE buffer and contained 0.5µg/ml EtBr (BioRad). Loading buffer was added to the DNA sample, to give a final concentration of 1x, before loading into the gel. Gels were run in Hybaid tanks containing 1x TBE at 50-100V. After electrophoresis, DNA fragments were visualized on a UV transilluminator and photographed using a video copy processor (Mitsubishi).

250ng of 1Kb DNA ladder (Boehringer Mannheim) was run on each gel to enable the size of DNA fragments to be determined approximately.

2.4.3 Purification of DNA from agarose gels

After electrophoresis, DNA fragments were visualized on a UV transilluminator and quickly excised using a sterile scalpel blade to avoid UV damage to the DNA. DNA

was then isolated from the gel slice using a QIAEX Gel Extraction Kit (Qiagen) as per manufacturers instructions.

2.5 DNA sequencing

2.5.1 Sequencing reaction

Double-stranded DNA templates were sequenced using the dideoxy-sequencing method (Sanger *et al.*, 1977) and the primers, (summarised in table 2.1)
In a total volume of 16µl, double-stranded DNA and sequencing primer were mixed to give a final concentration of 0.16µg/µl and 30ng/µl respectively. 4µl of 1M NaOH was added and the mixture incubated at RT for 5mins. 4µl of 2.5M ammonium acetate (adjusted to pH4.6 with acetic acid) was then added, followed by 55µl of ice cold ethanol. The reaction was then precipitated on ice for 15mins, pelleted by centrifugation for 15mins at 4°C, washed in 1ml of cold 70% (v/v) ethanol (all subsequent % ethanol solutions are v/v), dried under vacuum and resuspended in 8.75µl of dH_2O.

The sequencing reaction was performed using a Sequenase version 2.0 Kit (United States Biochemical). To the 8.75µl of template/primer, 1.25µl of DMSO, 2.5µl of sequenase reaction buffer, 1µl of dithiothreitol, 2µl of diluted labelling mix (1:5), 0.5µl of [α-P^{33}]-dATP (10µCi/µl) (Amersham) and 2µl of diluted sequenase (1:8) were added and the reaction incubated at RT for 5mins. During this time each termination mix was diluted with DMSO to give a final concentration of 10% and 2.5µl of each aliquoted into separate wells of a 96 well plate (Sero well). This plate was then warmed to 37°C in a water bath and 3.5µl of the completed sequencing reaction was added to each of the 4 wells. These samples were incubated in a 37°C water bath for 5 mins and then the reaction terminated by adding 4µl of the stop solution.

2.5.2 Electrophoresis and detection of sequencing reactions

The products of the sequencing reaction were separated by electrophoresis on vertical polyacrylamide-gels using BioRad apparatus. Prior to assembling the gel

apparatus, the glass plates and spacers were thoroughly cleaned and washed with ethanol. The plates were then sandwiched together, with the spacers maintaining a gap between the plates. The base was sealed by standing the glass plate in a tray containing 10mls of acrylamide (Severn Biotech), 50µl of TEMED (Gibco BRL) and 50µl of 25% (w/v) ammonia persulphate (APS). This solution was drawn by capillary action into the space at the base of the plates before setting. The gel was prepared by mixing 60mls of acrylamide (Severn Biotech), 68.6µl of APS. This was then poured between the two glass plates using a syringe and ensuring no air was trapped.

Before loading the samples, the gel was warmed to 50°C and the sequencing samples heated to 95°C for 3mins and then cooled on ice. 2µl of each sample was then loaded. The gel was run in TBE buffer, 1x in the bottom tray and 0.5x in the top, at 2.2KV. The gel was then removed from the plates, placed on 3mm filter paper (Whatman) and Saran wrap (Dow Chemical Company) placed on top. The gel was then dried at 60°C under vacuum on a Gel Dryer 583 (BioRad). The dried gel was then placed in a light-tight cassette with a signal enhancing screen and exposed to X-OMAT x-ray film (Kodak). Generally, a 12-16 hrs exposure time was sufficient. Films were then developed in an automatic x-ray film processor RGII (Fuji).

2.6 Isolation of DNA

2.6.1 DNA extraction from mouse tail tips and embryonic yolk sacs

2.6.2 Solutions

Lysis buffer: 100mM tris pH8.5, 5mM EDTA, 0.2% (w/v) SDS, 200mM NaCl

Tissue is added to 0.5mls lysis buffer and 50µl of a10x Proteinase K solution (1mg/ml) mixed and incubated at 55°C for 12-16 hrs. The solutions were then vortexed and spun in a bench top centrifuge for 10 mins. 0.4mls of the supernatant were taken and 0.5mls isopropanol added, mixed and centrifuged for 15 mins. The supernatant was removed and the pellet washed with 70% ethanol and spun in a

bench top centrifuge for 7 mins and then left to air dry for 15 mins. The DNA was then resuspended in 0.5mls of TE

2.7 Polymerase chain reaction (PCR protocols)

2.7.1 Oligonucleotides

Oligonucleotides (see table 2.1) were supplied by Genesys as precipitates and resuspended in an appropriate volume of dH_2O.

Table 2.1 Oligonucleotides used in tailtip PCRs and sequencing

Name	Sequence	Description	PCR conditions
I444	GAATGGACGCT GATAGATGAAT TG	Bacterial LacZ	94° 3mins + 30x (92°C 30 secs, 55°C 45 secs, 72°C 1 min) + 72°C 10mins
I443	AGCTCTGGCAC ACGTGTCAG	Bacterial LacZ	(same as above)
-2	AACCGTCGATA TTCAGCCATG	B-Gal, downstream	94°C 5mins + 35x (94°C 1min, 50°C 1 min, 72° 1 min) + 72° C 10mins
-1	GATTACCGTTG ATGTTGAAGT	B-Gal, upstream	(same as above)
bp1.2	ACGGTATCGAT AAGCTTCCTC	pTrp2-Msx2 mice upstream	94°C 3 mins + x35(94°C 20 secs, 65°C 20 secs, 72°C 1 min) + 72°C 5 mins
Msx2/3	CCTGACGCCAC GGACGCTC	pTrp2-Msx2 mice downstream	(same as above)
Msx2 back2	AGGAGCAGTCA GCAGAGTTG	Sequencing	50x 95°C 30 secs, 55°C 30 secs, 72°C 1 min
Intron pCi	TGCCTTCTCTCC ACAGGTGTC	Sequencing	(same as above)
Trp2 forward	TGGAAGACAAG GAGTAAAGTC	Sequencing	(same as above)

2.8 Transgenic methodology

2.8.1 Solutions

Micro-injection buffer: 10mM Tris, 0.1mM EDTA, pH7.5. Filter sterilised.

2.8.2 Methodology

The transgene was released from the vector by restriction digest and isolated by gel electrophoresis. The transgene DNA, eluted in micro-injection buffer, was then passed through a Micropure 0.22 column (Amincon) by centrifugation at 5000rpm in a bench top centrifuge, diluted in micro-injection buffer to 2ng/µl and finally passed through two 0.22µm Millex-GV4 filters (Millipore) to remove any particles.

Mice were superovulated by staff at the transgenic facility and oocytes collected by L. Marshall or L.McInnes. DNA at 2ng/µl was injected into the pronucleus of collected oocytes. Following overnight culture, those which reached the two cell stage were transferred into psuedopregnant host mice. Micro-injections and embryo transfers were performed by L.Marshall and L. McInnes

2.9 Analysis of mouse and chick embryos

2.9.1 Isolation of mouse and chick embryos

Mouse: The day the vaginal plug was observed after mating was designated E0.5. Pregnant females were killed by cervical dislocation and the embryos dissected from the uterus in ice cold PBS (Oxoid).

Chick: Fertilized Ross White eggs (Roslin, Edinburgh) were incubated on their sides in a 38°C humidified incubator. The embryos were dissected by puncturing the base of the egg and cutting a window so the embryo could be removed and transferred to ice cold PBS (Oxoid). Chick embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

2.9.2 Whole mount X-Gal staining of mouse embryos

2.9.3 Solutions

0.1M phosphate buffer: 126mls 0.1M $NaH_2PO_4.2H_2O$), 400mls 0.1M Na_2HPO_4 (pH7.3)

Fix: 2% (v/v) formaldehyde (added as Millory's 10% neutral buffered formalin-10% formaldehyde in 0.1M NaH_2PO_4), 0.2% (v/v) gluteraldehyde, 2mM $MgCl_2$, 5mM EGTA pH8 in 0.1M phosphate buffer.

Detergent wash: 2mM $MgCl_2$ 0.1% (v/v) sodium desoxycholate, 0.02% (v/v) Nonidet P40 (ICN), 0.05%(w/v) BSA (Sigma) in 0.1M phosphate buffer.

Stain solution: 0.085% (w/v) NaCl, 5mM $K_3Fe(CN)_6$ (Sigma), 5mM $K_4Fe(CN)_6$ (Sigma), 0.1% (w/v) X-Gal (Boehringer Mannhiem) in dimethyl formamide, made up in detergent wash.

4% (w/v) PFA: paraformaldehyde made up in PBS

2.9.4 Methodology

Isolated embryos were placed in fix for 1hr at 4°C and then washed 3x20mins at RT in detergent wash. Embryos were then incubated for 3-6hrs in stain solution at 37°C in glass containers. The embryos were then washed in 2x20mins changes of PBS and fixed in 4% PFA for 12-16 hrs.

Embryos were photographed and analysed using a Wild Heerbrugg microscope, mounted with a Photoautomat (Wild Leitz) camera using 64 ASA colour film (Fuji)

2.9.5 Wax embedding and sectioning of embryos

Embryos were fixed for 12-16hrs at 4°C in 4% PFA. Embryos were washed in PBS at 4°C for 30mins and then dehydrated by 1x15 mins change 30% ethanol and then 1x30 mins 50%, 2x30 mins 70%, 1x30mins 90%, 1x30mins 95%, 3x30mins 100%. The embryos were then transferred to glass dishes and washed with xylene for 1x15 mins at RT. Then they are washed for 1x15mins in xylene at 65°C.

Then embryos are then transferred to glass dishes and taken through 3 paraffin wax changes 3x30mins at 60°C. For embryos older than E10.5 the times for the ethanol and wax changes were increased to 45mins. After the final incubation in paraffin

wax, embryos were embedded in fresh wax in a plastic mould. This was then floated in a bath of cold water.

5 and 7μm sections were cut on a (Leitz) microtome. Sections were floated out in sterile water at 40°C and onto glass slides (Chance Propper). Slides were then incubated at 60°C overnight to seal onto sections. Slides were stored in a sealed box with silica gel desiccant. For *in situ* hybridization, sections were mounted on TESPA treated slides.

TESPA coating: Glass slides were washed in 10% (v/v) HCl in 70% ethanol for 20secs, then washed in sterile dH_2O for 20secs and finally washed in 100% acetone (filter sterilized) for 20 secs. The slides were then air dried, washed in 2% (v/v) TESPA (3-aminopropyl-triethoxysilane: Sigma) in acetone (filter sterilized) for 20secs and washed in 2x20secs changes of 100% acetone (filter sterilized). Slides were then air dried and stored in a sealed box.

2.9.6 H and E staining

Slides were dewaxed by placing in 2x5mins changes of xylene, then rehydrating in 2x5 mins changes of 100% ethanol followed by 5 mins changes of 90%, 70%, 50% and 30% ethanol. Slides were then washed for a few minutes in water. Slides were placed in haematoxylin (Surgipath) for 4-5mins, washed in running tap water and then differentiated in 1% (v/v) HCl in 70% ethanol for a few seconds. The slides were then washed in running tap water and transferred to lithium carbonate solution for a few seconds. The slides were then washed in running tap water, stained in eosin (3parts 1% (v/v) aqueous eosin (Surgipath), 1 part 1% (v/v) ethanol and 0.05% (v/v) acetic acid) for 1-2 mins and rinsed in water. The slides were then processed as follows: 1x15 secs in 100% ethanol, 2x 1 mins in 100% ethanol and 1x 5 mins in 100% xylene. The slides were then transferred to fresh xylene and mounted with a glass coverslip (Chance Propper) in DPX.

Slides were analysed and photographed with a Ziess Axioplan 2, mounted with a Yashica 108 multiprogram camera using 64 ASA colour film (Fuji).

2.9.7 mRNA radioactive *in situ* hybridization

2.9.8 Solutions

Proteinase K buffer: 50mM Tris, 5mM EDTA (pH 8.0).
Hybridization Mix: 50% (v/v) formamide, 10% (v/v) dextran sulphate, 1x Denhardts, 20mM Tris (pH8), 0.3M NaCl, 5mM EDTA, 10mM sodium phosphate, 0.5mg/ml tRNA and 50mM DTT (added immediately before use)
High Stringency Wash: 50% (v/v) formamide, 2x SSC, 50mM DTT (added immediately before use)
NTE: 0.5mM NaCl, 10mM Tris, 5mM EDTA, pH 7.5.

2.9.9 Radioactive labelling of riboprobes

The DNA probe template was linearised by digestion with the appropriate restriction enzyme and cleaned by phenol/chloroform purification and then using a Gene Clean Spin Kit (Bio 101) as per manufacturers instructions. Radiolabelled probes were prepared by transcription using T7 polymerase in the presence of ^{35}S rUTP as follows. 3µl of 10x transcription buffer (Boehringer Mannheim), 1µl of 10mM rATP, 1µl of 10mM rCTP, 1µl of 10mM rGTP, 1µl of 1M dithiothreitol (DTT), 3µl of dH2O, 12µl of 35S rUTP (>1mMCi/100µl: Amersham), 5µl of linearised DNA template (0.5-1µg/5µl), 1.2µl of RNase Inhibitor (Boehringer Mannheim) and 0.8µl of T7 polymerase (Boehringer Mannheim) were added in this order and incubated at 37°C for 25mins. A further 0.8µl of T7 polymerase was added the solution was mixed by flicking the side of the tube and the reaction incubated at 37°C for a further 25 mins. 2µl of 10mg/ml tRNA and 1µl of DNaseI (Boehringer Mannheim) was then added and the reaction incubated at 37°C for10 mins. Addition of 2µl of 100mM EDTA stopped the reaction.

The riboprobe was purified by adding 100µl of TE with 50mM DTT, and application of this mixture to a Microcon 30 filter (Amincon) and centrifuging for 15 mins in a bench top centrifuge. A further 100µl of TE with 50mM DTT was then spun through the column as before. Then 25µl of TE, 50mM DTT was then added to the micron and the column incubated on ice for 20 mins. The micron was then inverted into a fresh tube and the probe collected by centrifugation for 5 mins in a bench top centrifuge. This elution procedure was then repeated with a further 25µl of TE, 50mM DTT.

The incorporation efficiency and dpm/µl was calculated by taking 1µl of probe and adding 19µl of TE, 50mMDTT. This mixture was added to two Whatman GF/B filters (10µl/filter) and one filter washed 3x in TCA (trichloroacetic acid solution; Sigma) and once in 100% ethanol, drawn through under vacuum. The filters were then air dried and both the washed and unwashed filters counted in separate scintillation vials in 10mls of Ecolite Scintillation Fluid (ICN) using a Packard Tri-Carb 1500 Liquid Scintillation Analyser. % incorporation = (precipitated count)/(total count) x 100.

Table 2.2. Probes used for *in situ* hybridization

Plasmid	Description	Enzyme to linearise
G#8ps7	Chick *Msx2*, 700bp PstI fragment	BamHI
pλ26AR3	Mouse *Msx2*, 985-1371	BamHI
P5A7	Mouse *Trp2*, 1200bp EcoRI fragment	HindIII
pBSMitf	Mouse *Mitf*, 1350bp EcoRI fragment	XbaI

2.9.10 Prehybridization

Slides were dewaxed and rehydrated as follows in batches of 20: 2x 5mins in 100% xylene, 2x 2 mins in 100% ethanol, 2 mins in 90%, 70%, 50%, 30% ethanol and finally 1x 2 mins in PBS with agitation. The slides were then fixed in 4% PFA (pH7.3) for 10 mins, split into two racks and washed twice in PBS for 2 mins. The

slides were then incubated in proteinase K buffer with 20µg/ml Proteinase K (BCL Biochemicals) for 7.5 mins at RT. The slides were then transferred to PBS for 1 mins, 4% PFA for 2 mins, rinsed in sdH_2O for 10 secs, 0.1M triethanol amine (TEA) pH 8 for 30 secs, 2x 5mins 0.1M TEA containing 625µl/100mls of acetic anhydride (Sigma) with stirring, PBS for 2 mins and finally 0.85% (w/v) NaCl for 2 mins. The slides were then dehydrated as follows: 1 min changes in 30%, 50%, 70%, 90% ethanol, then 3x5 mins changes of 100% ethanol. The slides were then air dried and stored in a sealed box with silica gel desiccant.

2.9.11 Hybridization

^{35}S riboprobe was diluted with TE, 50mM DTT so that when hybridization mix was added at a ratio of 1:9 (probe to hybridization mix), the final count was 1.1×10^5 dpm/µl. The probe/hybridization mix was heated at 80°C for 2 mins, rapidly cooled on ice and then ≈50µl added to each slide. A glass coverslip was placed over each slide and the slides placed horizontally in a sealed box containing a tissue soaked in 5mls of 50% (v/v) formamide, 5x SSC. The box was heat sealed inside two plastic bags and submerged in a water bath at 55°C for 16-18 hrs.

2.9.12 Post hybridization washes

Slides were removed from the hybridization box and placed in 5x SSC, 10mM DTT for 20 mins at 55°C, this allows the coverslips to be removed. The slides were then transferred to a Hybaid Omnislide Wash Module containing high stringency wash at 65°C for 30 mins. The slides are then washed in 3x 10 mins changes of NTE at 37°C before being incubated in NTE containing 20µg/ml of RNAase A for 30 mins at 37°C. The slides were then washed in NTE at 37°C for 5 mins, then transferred back to the wash module containing high stringency wash at 65°C for 30 mins. The slides were then washed in 4x 10 mins changes of 2x SSC at RT followed by 4x5 mins changes of 0.1x SSC at RT. Finally, the slides were dehydrated by 1 mins changes in 30%, 50%, 70% and 90% ethanol followed by 2x 5 mins changes of 100% ethanol. The slides were then air dried.

2.9.13 Autoradiography

Using a S902 safety filter lamp, slides were dipped (two at a time, back to back) in 1:1 sdH_2O to Ilford K5 emulsion at 41°C. The slides were then allowed to dry slowly in a light tight box containing damp tissues for 12-16 hrs. The dried slides were then placed in a light-tight box with silica desiccant and stored at 4°C. Slides were then exposed to the film for 4-6 weeks and then developed by immersing in Kodak D19 developer for 4 mins, washed in sdH_2O for 10 secs, fixed in a 1:3 dilution of AMFIX: sd H_2O for 5 mins and then rinsed in 2x 10 mins changes of water. The slides were then counter stained in 1 % methyl green (Sigma), air dried and mounted with a glass coverslip in DPX.

2.10 Cell culture

2.10.1 Culture conditions for established cell lines

APRE19: DMEM/Nut. Mix F12 (Gibco BRL), 10% (v/v) FCS, 37°C, 5% CO_2.

2.10.2 Primary cultures of chick PRE

Fertilized Ross White eggs were incubated and embryos collected under sterile conditions as described previously. Fifteen 5-day-old chicks were routinely dissected at a time. The embryos were placed in ice cold PBS and the heads removed using flamed forceps. The heads were transferred to a fresh dish of ice cold PBS and the eyes removed using a flamed-tungsten needle and forceps under a Stemi 2000 (Zeiss) dissection microscope. A hypodermic needle was then used to hold the eye so the choriod fissure is upwards and the front of the eye is removed using a sterile scalpel blade. The inner neural retina is peeled away using flamed forceps. If the neural retina is required it can be transferred to a sterile tube using a sterile pipette and stored on ice. The PRE and associated perioptic mesenchyme was then placed in a dish containing 7.5mg/ml of filter-sterilised collagenase A (Boehringer Mannheim) in PBS and incubated at 37°C for 5 mins. The tissue was then rinsed 2x with ice cold

PBS. The PRE was then peeled away from the mesenchyme using flamed forceps and placed in a sterile tube, on ice, using a sterile flame-polished, glass pipette.

In a laminar flow air cabinet the excess PBS was removed from the collected PRE or neural retina. 2mls of trypsin/versene 1;10 (0.2% (w/v) trypsin, 0.04% (w/v) EDTA in Dulbecco 'A') was added and the cells incubated for 3 mins at RT with gentle shaking. 2mls of FCS was then added and the cells dissociated by briefly sucking up and down in a sterile, flame-polished, glass pipette. The cells were then pelleted by centrifugation at 1200 rpm for 5 mins, resuspended in 2 mls of appropriate media, re-pelleted by centrifugation at 1200 rpm for 5 mins and then resuspended in the desired volume of medium and typically seeded into 4 wells of a 24-well tissue-culture plate (Linbro, ICN). Cell were cultured at 37°C, 5% CO_2 in a humidified incubator, changing the medium every other day.

Cells were passaged by removing the medium and incubating in trypsin/versene 1:10 (0.2% (w/v) trypsin, 0.04% (w/v) EDTA in Dulbecco 'A') at RT for 5 mins or until most of the cells had detached from the dish. The cell suspension was then transferred to a sterile tube and the trypsin inactivated by adding an equal volume of FCS. The cells were then pelleted by centrifugation at 1200 rpm for 5 mins, resuspended in the appropriate medium, re-pelleted and finally resuspended in the desired volume of medium and seeded onto fresh tissue-culture plates.

Media

EMEMF was used routinely.

EMEMF: EMEM, 8% (v/v) FCS 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin.

NB27: Neurobasal, 1xB27, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin.

2.10.3 Transient transfection of eukaryotic cells

For cells grown in 30mm well plates: 1μg of DNA was diluted in 100μl of Optimem1 with glutamax 1 (Gibco BRL) and 12μl of Lipofectamine (Gibco BRL) mixed with 88μl of Opitmem 1 with Glutamax 1 (Gibco BRL). The diluted DNA and Lipofectamine were mixed together and incubated at RT for 30mins before adding 800μl of Optimem1 with glutamax 1 (Gibco BRL). The cells were rinsed with Optimem1 with glutamax 1 (Gibco BRL) and 1ml of diluted DNA/Lipofectamine mix added to each well. The cells were then incubated with this mixture for 5 hrs in a 37°C, 5% CO_2 tissue-culture incubator before removing the transfection mixture rinsing and replacing with appropriate media.

For cells grown in 8-well chamber slides: 1μg of DNA was diluted in 100μl of Optimem 1 with glutamax 1 (Gibco BRL) and 12μl of Lipofectamine reagent mixed with 88μl of Optimem 1 with glutamax 1 (Gibco BRL). The dilute DNA and Lipofectamine were mixed together and incubated at RT for 30 mins before adding 1.6mls of Optimem 1 with glutamax 1 (Gibco BRL). The cells were rinsed in Optimem 1 with glutamax 1 (Gibco BRL) and 150μl of diluted DNA/Lipofectamine mix added to each well. The cells were then incubated with this mixture for 5 hrs in 37°C, 5% CO_2 incubator before replacing with the appropriate medium.

2.10.4 X-Gal staining of cultured cells

Solutions

Fix: 0.5mls formaldehyde (filter sterilized), 0.08mls 25% (v/v) glutaradehyde, 9.42mls PBS

Stain 0.02g potassium ferrocyanide (sigma), 0.016g potassium ferricyanide (Sigma), 20μl 1M $MgCl_2$, 9.75mls PBS, 0.25mls 40mg/ml X-Gal (Melford) in DMSO.

Methodology

Cultures were rinsed in PBS and fixed for 5mins at 4°C in fix. The cells were then rinsed in PBS and the stain solution added and incubated in the dark at 37°C for 3hrs. The stain solution was removed and the cells rinsed in PBS.

Cells were counted and photographed using a Labovert FS (Leitz) inverted phase microscope mounted with a photoautomat (Wild Leitz) camera using 64 ASA colour film (Fuji). Cells were counted by recording the number of cells in the field of view at x10 magnification. The dish was then moved horizontally to another area of the dish.

2.10.5 Immunofluorescent staining of cultured cells

Cells were cultured in glass chamber slides. Cells were first rinsed with ice cold PBS and then fixed for 10 mins at RT in -20°C 1:1 methanol:acetone. The cells were then rehydrated for 30mins in PBS and blocked for 1 hour at RT in blocking solution (PBS containing 6.7% (v/v) glycerol, 2% (w/v) BSA (Sigma) and 0.2% (v/v) Tween 20 (Sigma). The plastic chambers were then removed leaving the cells on the slide. Optimal dilutions of the primary antibodies were made in blocking solution and then applied to the cells for 1 hr at RT (See table 2.3). The cells were then washed for 3 x 5mins changes of PBS, 0.1% (v/v) Tween 20 and incubated for 1 hr in the dark at RT with either fluoresein (FITC) or Texas-red conjugated secondary antibodies diluted in blocking solution. Following this the slides were washed 3 x 5mins changes of PBS, 0.1% (v/v) Tween 20 and mounted with a glass coverslip in Vectashield mounting medium containing DAPI (Vector Laboratories). The coverslip was sealed on with rubber solution (Pang) and observed with a fluorescence microscope.

To control for non -specific binding the secondary antibodies were applied to cells incubated with the wrong primary antibody. Some background staining was sometimes observed in these controls.

Table 2.3 Antibodies used in immunohistochemistry

Antibody	Description	Working dilution	Supplier
Mouse anti-βGal	Reacts with E.coli βGalactosidase	1:200	Promega
Rabbit anti-βGal	Reacts with E.coli βGalactosidase	1:200	5 Prime to 3 Prime
Donkey anti-rabbit IgG	Fluorescein (FITC) conjugated secondary Ab	1:200	Jackson ImmunoResearch laboratories
Donkey anti-mouse IgG	Texas Red conjugated secondary Ab	1:200	Jackson ImmunoResearch laboratories
Rabbit anti-Mitf	Reacts to mouse Mitf	1:200	Gift from Dr. Arnheiter (Opdecamp *et al.*, 1997)
Mouse anti-PCNA	Reacts to PCNA	1:200	Santa Cruz Biotechnology

2.11 mRNA DIG in situ hybridization

2.11.1 Solutions

Prehybridization buffer: 50% (v/v) dionized formamide

5x SSC pH 4.5 (pH with 1M Citric acid), Heparin 50µg/ml, tRNA 100µg/ml, 0.1% (v/v) TritonX-100

PBT: PBS + 0.1% (v/v) TritonX-100

TBST: 0.14M NaCl, 2.7mM KCl, 0.025M Tris HCl pH 7.5, 0.1% (v/v) Triton X-100

NTMT: 100mM NaCl, 100mM Tris HCl pH9.5, 50mM MgCl2, 0.1% (v/v) TritonX-100

NBT/ BCIP: nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate.

2.11.2 Preparation of cells

Dedifferentiated chick PRE cells from a 5day old chick were plated out onto 25mm TESPA coated coverslips in 0.5mls of DMEM and left to adhere for 45 mins at 37°C and then the well was flooded with DMEM. The following day the cells were transfected as described for 30mm wells. After 24 hrs the cells were fixed for 12-16hrs in 4% PFA in PBS at 4°C.

The cells were then washed in PBS and dehydrated for 5 mins each solution of 25%, 50%, 75% methanol in PBT and 2 x 5 mins100% methanol. The coverslips can be stored at this stage at -20°C in 100% methanol.

As a control for endogenous Msx2 activity the AER and anterior mesenchyme of ten late E4 chicks was dissected using a sterile scalpel blade. The dissected tissue was then transferred to a dish containing 7.5mg/ml of filter-sterilised collagenase A (Boehringer Mannheim) in PBS and incubated at 37°C for 5 mins. In a laminar flow air cabinet the excess PBS was removed from the tissue and 2mls of trypsin/versene 1:10 (0.2% (v/v) trypsin, 0.04% (w/v) EDTA in Dulbecco 'A') was added and the cells incubated for 3 mins at RT with gentle shaking. 2mls of FCS was then added and the cells dissociated by briefly sucking up and down in a sterile, flame-polished, glass pipette. The cells were then pelleted by centrifugation at 1200 rpm for 5 mins, resuspended in 2 mls of appropriate media, re-pelleted by centrifugation at 1200 rpm for 5 mins and then resuspended in the desired volume of medium and typically seeded onto 4 sterile TESPA coated coverslips. The coverslips were incubated for 1 hour at 37°C to allow the cells to adhere. Then the cells were fixed for 12-16hrs in 4% PFA in PBS at 4°C and dehydrated as described.

2.11.3 DIG labelling of riboprobes

The DNA probe template was linearised by digestion with the appropriate restriction enzyme and cleaned twice using a Gene Clean Spin Kit (Bio 101) as per manufacturers instructions.

DIG labelled probes were prepared by transcription using T7 polymerase in the presence of DIG UTP using a DIG RNA Labelling Kit (Boehringer Mannheim). The reaction was set up as follows; 5µl of linearised DNA template, 2µl of 10x DIG RNA labelling mix (Boehringer Mannheim), 2µl of 10x transcription buffer (Boehringer Mannheim), 9µl of sterile RNAase free dH_2O, 2µl of RNA T7 RNA polymerase (Boehringer Mannheim) were added in this order, mixed and incubated for 2 hrs at 37°C. Then 2µl of RNAase free DNAaseI was added and incubated for 15 mins at 37°C. Then on ice 2µl of 0.2M EDTA solution (Boehringer Mannheim) was added.

2.11.4 Prehybridization

Prehybridization buffer: 50% (v/v) dionized formamide
5x SSC pH 4.5 (pH with 1M Citric acid), Heparin 50µg/ml, tRNA 100µg/ml, 0.1% (v/v) TritonX-100. Coverslips were rehydrated as follows: 2x 5mins in 100% methanol, 5 mins in 75%, 50%, 25%, methanol. They were then washed 3x 3 mins in PBT. To permeabilise the cells they were washed for 3 mins in Proteinase K (Boehringer Mannheim) (10µg/ml in PBT) and then 5 mins in filter sterilised Glycine (Boehringer Mannheim)(2µg/ml in PBT) and then washed 2 x 5 mins in PBT. The cells are then fixed for 20 mins in freshly prepared 4% PFA/0.2% gluteraldehyde. The cells were then washed 3x 5 mins with PBT and then washed 1:1 PBT:prehyb buffer for 5 mins. Finally the cells were washed for 5 mins in prehyb buffer and then incubated with prehyb buffer for 1 hour at 70°C in sealed humidified chamber (using filter paper soaked in 4x SSC)

2.11.5 Hybridization

The DIG labelled probe is diluted 1:500 with prehyb buffer and applied to the cells and incubated for 12-16 hrs at 70°C in a sealed humidified chamber.

2.11.6 Post hybridization washes

The hybridization solution was removed and the cells washed 2x 5 mins at 70°C with 50% (v/v) formamide, 2x SSC, 0.1% (v/v) TritonX-100. They were then washed 2x 20 mins at 70°C in the same solution. Following this the cells were washed 2x20mins at 70°C with 0.2xSSC, 0.1% (v/v) TritonX-100. The coverslips were then allowed to come up to RT and then they were washed 3 x 5 mins in TBST at room temperature. The cells were then blocked for 1 hr in 10% (v/v) heat inactivated sheep serum/ TBST at RT. The cells were then incubated 12-16 hrs at 4°C in 1ml of 1:2000 dilution of alkaline phosphatase-conjugated anti-digoxigenin antibody, Fab fragments in 1% (v/v) sheep serum in TBST

2.11.7 Detection

The coverslips were washed 2x5 mins TBST, followed by 3x45 mins in TBST at RT. Then they were washed 2x5 mins in NTMT at RT. For the colour reaction the cells were incubated with freshly made up 4.5µlNBT/3.5µl BCIP per ml NTMT in the dark. After incubating for approximately 2 hrs the colour had fully developed and the reaction was stopped by washing in PBS (at pH 5.5 or less) with 1% (v/v) TritonX-100 for at least 10 mins at 4°C. The cells were then fixed for 20 mins with 4%PFA/0.1% (v/v) gluteraldehyde and stored in PBS.

Chapter 3 The application of an *in vitro* assay to investigate the cellular functions of *Msx1* and *Msx2*

3.1 Introduction

The *in vitro* assay for Msx cellular function examined in this chapter has the potential to provide a controlled, physiologically relevant system in which to investigate the genes regulated by the Msx proteins and their effects on cellular processes. In the assay a small proportion of primary chick PRE cells transfected with a construct expressing mouse *Msx*2 show an altered phenotype, with two or more dendritic-like processes. The cells forming dendritic cells are not contaminating mesenchyme cells, (Holme, 1998). These results suggest ectopic expression of mouse *Msx*2 may promote neural cell characteristics in PRE cells. The expression pattern of mouse *Msx2* in the eye is consistent with this hypothesis and suggests that *Msx2* could function in the processes that pattern neural fate or suppress pigmented cell fate in the prospective NR domain of the optic neuroepithelium. In this chapter I have investigated the practical application of the *in vitro* assay to explore Msx cellular function. The fact that only a small number of cells exhibit an *Msx*2-induced change is a severe limitation to the usefulness of the assay. The aims of the present work are; to increase the number of cells displaying an *Msx*2-induced change and use the assay to investigate the cellular functions of the Msx proteins. In addition, I have used the assay in a top-down approach to investigate the effect of *Msx2* transfection on the cellular process of cell division. I have also explored the feasibility of applying a bottom-up approach, using DIG-*in situ* hybridization to assay Msx candidate downstream target genes in the *in vitro* cellular assay. By ectopically expressing mouse *Msx1* in the *in vitro* assay I have used it to investigate potential functional redundancy between mouse *Msx1* and *Msx2* at the cellular level.

3.2 The cellular effects of ectopic mouse Msx gene expression in chick PRE cells

3.2.2 m*Msx2* expression in dedifferentiated PRE cells promotes formation of the dendritic phenotype

A small proportion, 0.98% of m*Msx2*-transfected primary chick PRE cells, show a dendritic phenotype, no cells with this phenotype were observed in control-transfected primary cultures. This suggests that a cellular effect of ectopic mouse *Msx2* expression, in a small number of chick PRE cells, is induction of neural characteristics. The small number of PRE cells that can be dissected from a chick for primary cultures and the small proportion of cells that show a detectable change as a result of m*Msx2* expression are severe limitations on the use of the assay to further investigate *Msx2* cellular functions. In culture, chick PRE cells divide and after 2-3 days in culture they begin to dedifferentiate and lose their pigmentation. This state of dedifferentiation can be maintained by regular passaging of the PRE cells every 2 days. The following experiment was done to investigate whether m*Msx2* promotes the formation of dendritic cells from dedifferentiated PRE cells, potentially increasing the amount of starting material for the assay. In addition, ectopic *Msx2* expression may be inducing the dendritic phenotype by promoting PRE cell dedifferentiation and using dedifferentiated PRE cells may increase the number of transfected cells developing the dendritic phenotype. Finally, culture conditions may be promoting or inhibiting the formation of dendritic cells. Parallel cultures of dedifferentiated PRE cells were set up to test the influence of using the neural substrate laminin and neural basal media (Brewer *et al.*, 1993) on the proportion of m*Msx2*-transfected dedifferentiated PRE cells acquiring a dendritic phenotype.

Primary PRE cells from 30, 5d chicks were dedifferentiated, by culturing them for 10 days and passaging a total of 5 times. These cells were seeded on to plastic or laminin-coated tissue culture dishes and cultured in standard and NB27 media (Brewer *et al.*, 1993), respectively, overnight. The dedifferentiated cells were transfected with m*Msx*2 and control constructs and cultured in their respective media. 48hrs after transfection the cells were fixed and stained for β-Galactosidase

activity, the results of this preliminary experiment are shown in Tables 3.1 and 3.2 and Figs 3.1, 3.2 and 3.3.

Table 3.1 Two duplicate counts in independent wells of proportion of m*Msx2*- and control-transfected dedifferentiated PRE cells with a dendritic morphology under standard culture conditions. The transfected cells counted represent an estimated 70% of the total number of transfected cells.

	Plastic and DMEM			
	Construct transfected			
	Msx2	control	*Msx2*	control
Number of transfected cells counted	1021	1073	1009	1119
Number with a dendritic morphology	41	0	52	0
% of transfected cells with a dendritic morphology	4	0	5.1	0

Table 3.2 Two duplicate counts in independent wells of proportion of m*Msx2*- and control-transfected dedifferentiated PRE cells with a dendritic morphology under neural-specific culture conditions. The transfected cells counted represent an estimated 70% of the total number of transfected cells.

	Laminin and neural basal media			
	Construct transfected			
	Msx2	control	*Msx2*	control
Number of transfected cells counted	1012	1053	1024	1006
Number with a dendritic morphology	52	0	55	0
% of transfected cells with a dendritic morphology	5.1	0	5.4	0

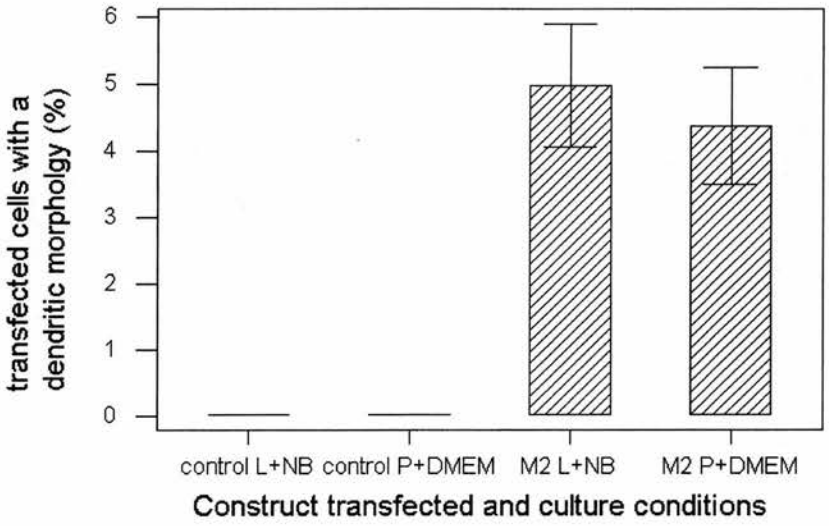


Fig. 3.1 Percentage of m*Msx2*- and control-transfected cells with a dendritic morphology on plastic and standard media and on laminin and neural-specific media.

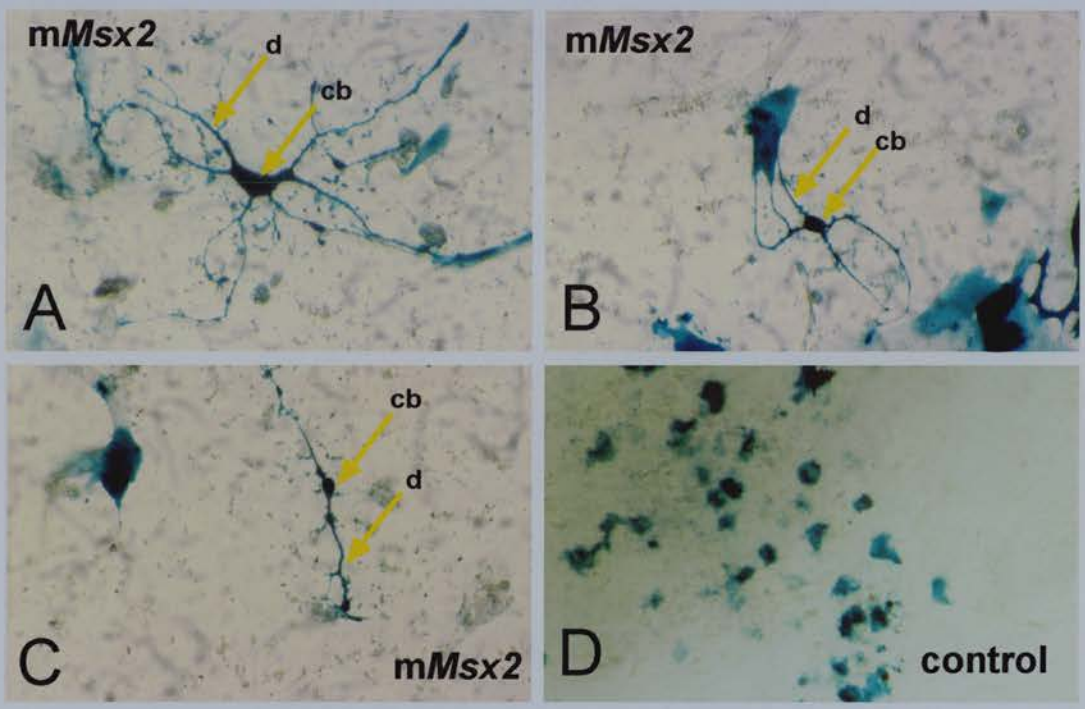
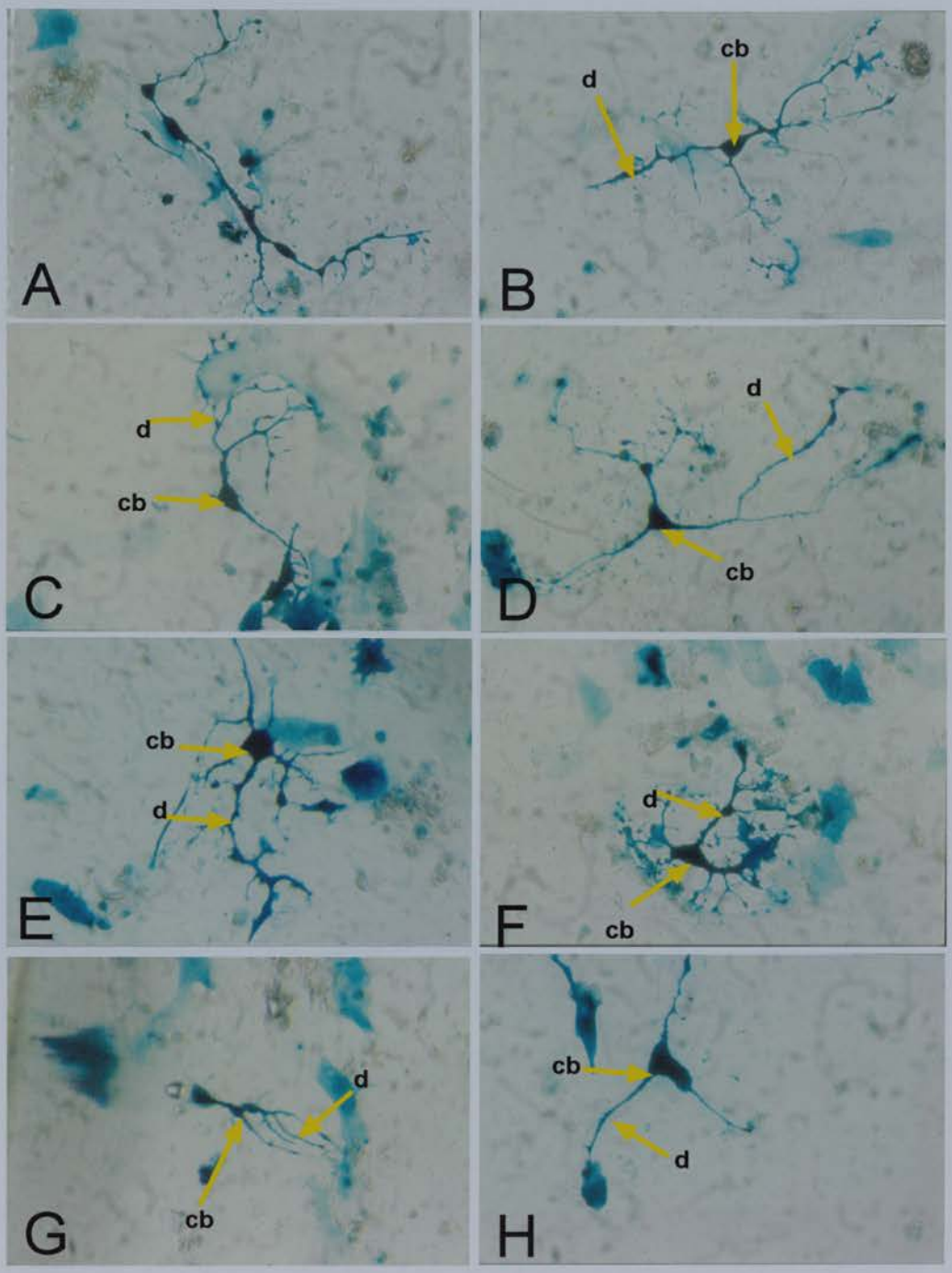


Fig. 3.2 Examples of dendritic cell phenotype of m*Msx2*-transfected cells and control transfected cells on plastic and in DMEM.Dedifferentiated PRE cells from 5d chick were transfected with either m*Msx2* or control construct, cultured for 48hrs, fixed and stained for BGal. A, a m*Msx2*-transfected cell with a highly branched morphology, x25. B, a m*Msx2*-transfected cell with multiple processes extending from the cell body, some making contact with a neighbouring m*Msx2*-transfected cell, x25. C, a m*Msx2*-transfected cell with a bipolar morphology, with two processes extending in opposite directions from the cell body, x25. D, control-transfected cells with regular PRE cell morphology, x4. d=dendrites and cb=cell body.

Fig 3.3 (Opposite), Further examples of m*Msx2*-transfected with dendritic phenotypes on different substrates and media. Dedifferentiated PRE cells from 5d chick were transfected with m*Msx2* construct, cultured for 48hrs, fixed and stained for β-Gal. A, C, E and G were grown on plastic and in DMEM. A, dendritic cell with no clear cell body, x25. C, dendritic cell with several processes potentially making contact with neighbouring cells, x25. E, dendritic cell with multiple processes emanating from the cell body, x25. G, dendritic cell with several processes apparently growing towards a neighbouring cell, x25. B, D, F and H were grown on laminin and in NB27 media.

B, dendritic cell with several processes, x25. D, dendritic cell with several processes, x25. F, dendritic cell with several highly branched processes, x25. H, dendritic cell with several processes, x25. d=dendrites, cb=cell body.


A
B
cb
d
C
d
cb
D
d
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E
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F
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G
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H
cb
d

The preliminary results shown in tables 3.1 and 3.2 and Fig 3.2 show that a proportion of m*Msx2*-transfected dedifferentiated PRE cells do form a dendritic phenotype. Using dedifferentiated PRE in 4 independent experiments, originally from 4d and 6d old chicks, subsequently gave similar results (Holme, 1998). Thus, ectopic expression of m*Msx2* in dedifferentiated PRE cells, as in primary PRE cells, can promote formation of a dendritic morphology in a small number of transfected cells. The cellular effects of ectopic m*Msx2* expression in both dedifferentiated and primary PRE cells appear to be similar. This could be investigated by using the antibody to class β-tubulin which has been shown to be upregulated in m*Msx2*-transfected primary PRE cells (Holme *et al.*, 2000). Dedifferentiated PRE cells can be used in further experiments with the *in vitro* assay, increasing the number of cells available to investigate the cellular functions of the Msx proteins.

The chi-squared test on this preliminary data suggests laminin substrate and neural basal media does not significantly effect the proportion of m*Msx2*-transfected cells becoming dendritic (χ^2= 0.988, d.f.=1, p=<0.5). In addition, the results from a preliminary time course experiment, to examine whether laminin substrate and neural basal media increase the time which dendritic cells could be cultured post-transfection, suggested that these neural specific culture conditions does not improve the survival time of dendritic cells (data not shown).

The formation of the dendritic phenotype in *Msx2*-transfected cultures may require loss of some PRE characteristics. In the above experiment no control-transfected dedifferentiated PRE cells had a dendritic phenotype. However, interestingly, 2 or 3 untransfected cells in control-transfected dedifferentiated PRE cultures have a dendritic phenotype, under normal culture conditions. Dendritic cells were never observed in control-transfected primary PRE cultures. This suggests that on dedifferentiation a small number of PRE cells may be able to form dendritic cells in culture, independently of ectopic *Msx2* expression. Ectopic expression of m*Msx2* may be leading to the development of the dendritic phenotype by promoting dedifferentiation of the PRE cells. Using dedifferentiated PRE cells in the assay may increase the proportion of *Msx2*-transfected cells displaying a dendritic phenotype. In

three independent experiments with primary PRE cells an average of 0.98% (+/- 0.39%) m*Msx2*-transfected cells had a dendritic morphology (Holme, 1998). This can be compared with the results of the preliminary experiment with m*Msx2*-transfected dedifferentiated PRE cells under normal culture conditions the mean proportion of m*Msx2*-transfected cells developing a dendritic phenotype is 4.55% (+/- 0.78). This comparison suggests that the proportion of cells with a dendritic morphology may increase when dedifferentiated PRE cells are used. However, as presented later in this chapter, the proportion of dedifferentiated PRE cells developing a dendritic phenotype varies considerably between different experiments.

3.2.3 m*Msx2* expression in PRE cells produces cells with a dendritic morphology in serum-free culture conditions

The dendritic cells were first observed in standard culture conditions with media containing Fetal Calf Serum, which may contain unknown growth factors. These factors are not sufficient to cause PRE cells to adopt a neural morphology, since no neural-like cells were observed in primary control cultures, but it is possible that they may be required to support Msx2 function. The following experiments were conducted to test whether unknown growth factors in the serum contribute to the formation of dendritic cells. In two independent experiments cells from two cultures of dedifferentiated PRE cells, from 5d chicks, were transfected with either m*Msx2* or the control expression constructs and grown in serum-free conditions. 48 hrs after transfection the cells were fixed and stained for β-Galactosidase activity the data is shown in table 3.3 and Figs 3.4 and 3.5.

Table 3.3 Proportion of *Msx2*- and control-transfected dedifferentiated PRE cells with a dendritic morphology in two independent experiments under serum-free culture conditions. The number of transfected cells counted represents an estimated 70-80% of the total number of transfected cells.

	Experiment no.					
	1		2		**Mean**	
	Construct transfected					
	Msx2	control	*Msx2*	control	***Msx2***	**control**
Number of transfected cells	991	525	811	450	**1802**	**975**
Number with a dendritic morphology	21	0	9	0	**30**	**0**
% of transfected cells with a dendritic morphology	2.1	0	1.1	0	**1.7**	**0**

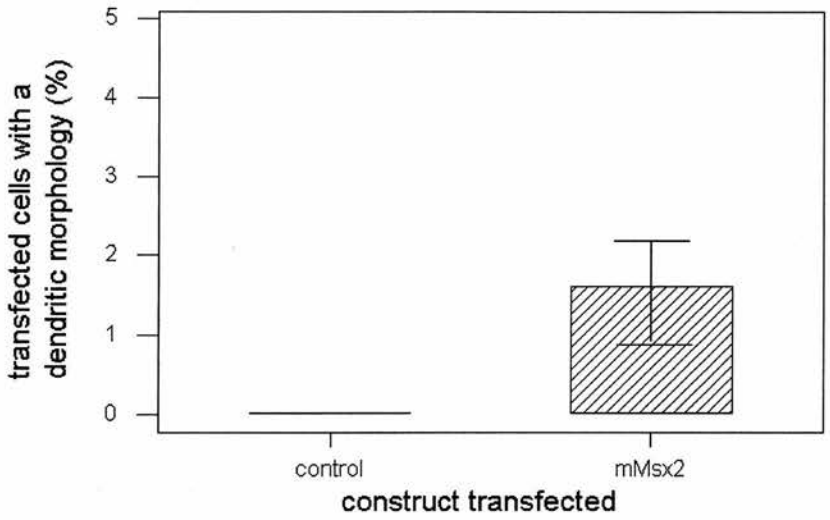


Fig. 3.4. Percentage of m*Msx2*- and control transfected cells with a dendritic morphology under serum-free culture conditions.

The results in table 3.3 and Figs 3.4 and 3.5 show that in serum-free conditions, 1.7% (+/- 0.72) of m*Msx2*-transfected PRE cells, had a dendritic morphology. No cells with a dendritic morphology were observed in the control-transfected cultures in serum-free culture conditions. Thus, ectopic *Msx2* expression promotes the formation of dendritic cells in chick PRE cells independently of growth factors in the serum component of the culture media. However, this experiment does not exclude the possibility that unknown serum growth factors may be contributing to dendritic cell formation. In a previous experiment, under normal serum-containing culture conditions, an average of 4.58% (+/- 0.78) m*Msx2*-transfected cells had a dendritic morphology. Under serum-free culture conditions, in comparison, 1.7% (+/- 0.72) m*Msx2*-transfected cells had a dendritic morphology. This difference may be a result of differences between the two PRE cell cultures or growth factors in the serum may be contributing to the formation of the dendritic phenotype. To test the influence of serum growth factors on the proportion of m*Msx2*-transfected cells developing a dendritic morphology it would be interesting to repeat this experiment with parallel

cultures of m*Msx2*- and control-transfected PRE cells in serum and serum-free culture conditions.

In addition, these experiments do not assay the influence of growth factors released by the PRE cells themselves. As described earlier, the processes of dendritic cells have been observed extending towards and making contact with both transfected and untransfected neighbouring cells (see Figs. 3.1 and 3.2), this may indicate that the dendritic-like processes are extending towards neighbouring cells, which are releasing growth factors. Dedifferentiating chick PRE cells in culture produce and secrete growth factors, including FGF1 and FGF2, (Guillonneau *et al.*, 1997). It has been reported that both FGF1 and FGF2 promote "transdifferentiation" of PRE into NR *in vivo* and *in vitro* (Guillemot and Cepko, 1992; Park and Hollenberg, 1989; Pittack *et al.*, 1991). But, it is not clear whether this process involves dedifferentiation and then redifferentiation. The chick PRE cells in the assay may be releasing FGF1 and FGF2, which promote transdifferentiation of PRE to neural retina in a few PRE cells. Antibodies to FGF1 and FGF2 could be used to investigate whether the PRE cells in the assay express these proteins. To develop the dendritic phenotype PRE cells may need to receive a threshold amount of growth factor so it would be interesting to investigate how addition of FGF1,2,8, EGF, BMP4 effects the number and morphology of dendritic cells.

In conclusion, the formation of m*Msx2*-transfected cells with a dendritic morphology is not dependent on or supported by growth factors in the serum. But, serum growth factors and/or factors released by the PRE cells themselves may contribute to the formation of dendritic cells. The formation of dendritic cells in m*Msx2*-transfected dedifferentiated or primary PRE cells may represent a cellular function of *Msx2* and can be used as an assay to investigate Msx protein function.

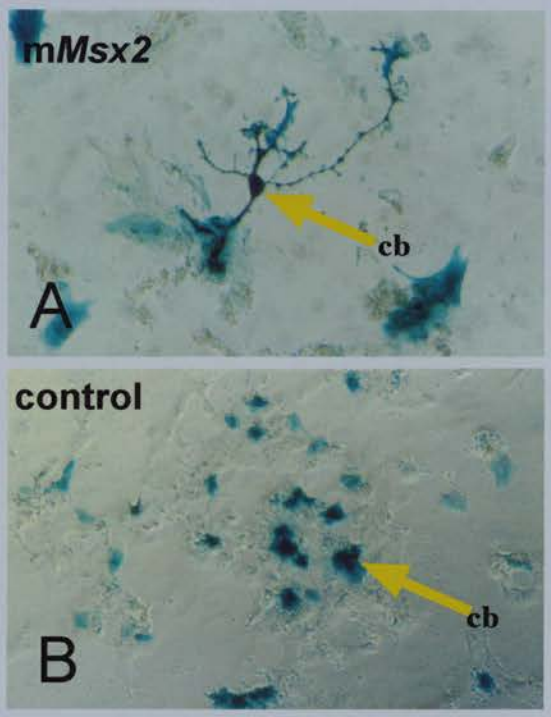


Fig3.5 m*Msx2*-transfected cells with a dendritic morphology observed under serum-free culture conditions. Dedifferentiated PRE cells from 5d chick were cultured in serum-free media, transfected with either m*Msx2* or control construct, cultured for 48hrs, fixed and stained for BGal. A, a m*Msx2*-transfected cell with multiple processes extending from the cell body, x25. B, a control-transfected with regular PRE cell morphology, x4. cb=cell body.

3.3 Cell division

A cell continues to divide until it terminally differentiates and the processes of cell division and differentiation are inter-related. The pathways downstream of the signals promoting cell division and differentiation may be linked (see reviews; Norton *et al.*, 1998; Schwartz and Baron, 1999). Msx1 and Msx2 may have roles stalling cellular differentiation in several different progenitor cell populations, including muscle, dermal and skull bone progenitors during development (Houzelstein *et al.*, 1999;Bendall *et al.*, 1999; Houzelstein *et al.*, 2000; Kim *et al.*, 1998; Satokata *et al.*, 2000; Satokata and Maas, 1994; Wilkie *et al.*, 2000). The period of *Msx* gene expression and stalled differentiation in these progenitor cell populations coincides with a period of active cell proliferation (Houzelstein *et al.*, 1999; Houzelstein *et al.*, 2000). Furthermore, BrdU-labelling in *Msx2*-null mice showed half the number of proliferating skull osteoprogenitors present in the osteogenic fronts compared to wildtype (Satokata *et al.*, 2000). Suggesting that Msx2 is involved in skull osteoprogenitor proliferation. In *Drosophila* carrying a mutation in *msh*, the *Drosophila Msx* homolog, cell proliferation was defective in the neural and muscle precursors cells which express *msh* (Isshiki *et al.*, 1997; Nose *et al.*, 1998). Recent results in progenitor cell lines *Msx1* and *Msx2* prevent exit from the cell cycle, but do not have a direct effect on cell division (Hu *et al.*, 2001). A role in the regulation of cell proliferation may be a conserved cellular function of the Msx/msh proteins.

Interestingly, the location of expression of mouse *Msx1* and mouse and chick *Msx2* in the ciliary margin, coincides with the location of a small population of recently discovered actively proliferating retinal stem cells (Fischer and Reh, 2000; Holme, 1998; Monaghan *et al.*, 1991). No expression of *Msx1* has been detected by *in situ* hybridization in the chick ciliary margin (Davidson, personal communication). *Msx2* may have a role maintaining the proliferative state of the retinal stem cells in chick and mice.

To investigate the effect of ectopic m*Msx2* on PRE cell division, triplicate wells of 5d chick primary PRE cultures were transfected with m*Msx2* or the control expression constructs. Proliferation was assayed using Proliferating Cell Nuclear Antigen (PCNA), an antibody which binds to a polymerase δ associated protein synthesized in early G1 and S phases of the cell cycle and was originally discovered as an antigen found only in the nucleus of dividing cells (Miyachi *et al.*, 1978). Based on an estimated cell cycle time of 24hrs, it was decided that 48hrs after transfection was a suitable time to assay for the effect of *Msx2* expression on PRE cell division. 48 hrs after transfection the cells were fixed for immunohistochemistry and antibodies for PCNA and βGal applied, followed by the corresponding secondary antibodies.

To calculate the basal proportion of actively dividing PRE cells in culture cells in untransfected and *Msx2*-transfected cultures were scored for PCNA expression, tables 3.4 and 3.5.

Table 3.4. Proportion of primary chick PRE cells in untransfected cultures actively dividing as assayed by expression of PCNA.

Construct transfected	Well no.	Total number of cells	Total PCNA +ve	Total PCNA -ve	unknown	**% of total cells PCNA+ve**
Untransfected	1	146	135	11	0	
	2	166	149	16	1	
	3	127	122	5	0	
	Total	**439**	**406**	**32**	**1**	**92**

The mean basal proportion of actively dividing cells in the 5d primary chick PRE cells in culture is 92% (+/- 3.3%), table 3.4. PCNA does not label dividing cells in G2 or M phases of the cell cycle and this may account for the small number of cells which do not appear to be actively dividing. To analyze the effect of *Msx2*-

transfection on cell proliferation control and *Msx2*-transfected cells in the same triplicate primary cell cultures were assayed for active cell division. βGal expressing cells in m*Msx2*-transfected and control-transfected cultures were assayed for PCNA expression, table 3.5, Fig 3.6 and Fig 3.7. As controls for cross-reactivity, between the secondary antibodies, one well was incubated with either anti-rabbit βGal or anti-mouse PCNA. These wells were then incubated with the non-complementary secondary anti-body, either anti-mouse FITC or anti-rabbit Texas Red. No fluorscent signal was detected in either of these wells (data not shown) indicating no cross-reactivity between the secondary anti-bodies.

Table 3.5. *Msx2*- or control-transfected 5d primary PRE cells assayed for active cell division using expression of PCNA. The number of cells counted represent an estimated 10% of the total number of cells transfected.

Construct transfected	Well no.	Total number of βGal+ve cells	Total PCNA +ve	Total PCNA -ve	unknown	**% of total cells PCNA+ve**
control	1	99	82	11	6	
	2	98	77	13	8	
	3	98	75	20	4	
	Total	**295**	**234**	**44**	**18**	**79**
Msx2	1	100	77	18	5	
	2	98	72	15	11	
	3	98	78	14	6	
	Total	**296**	**227**	**47**	**22**	**77**

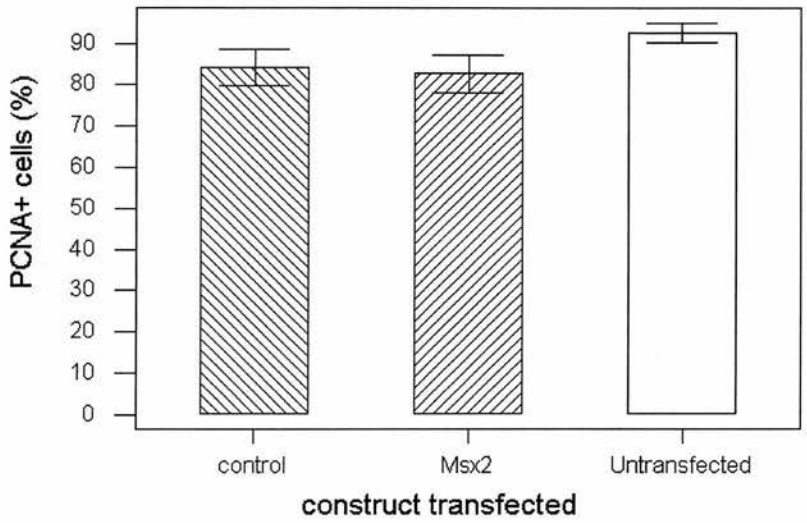


Fig.3.6. Percentage of untransfected, m*Msx2*- and control-transfected cells *PCNA*+. Cells classed as unknown were considered equally likely to be *PCNA*+ or *PCNA*- and are not included in the bar chart.

In m*Msx2*-transfected cells the average proportion of cells actively proliferating is 77% (+/- 3.1%), in control-transfected cells it is 79% (3.2%), table 3.5 and Fig 3.6. The chi-squared test indicates that there is no significant difference in the proportion of actively proliferating cells between m*Msx2*- and control-transfected cultures (χ^2= 0.176, d.f.=1, p=0.675).

The proportion of actively dividing cells in an untransfected PRE culture is 92% (+/- 3.3%), compared with 77% (+/- 3.2%) and 79% (+/- 3.1%) in *Msx2*- and control-transfected cells respectively, tables 3.4 and 3.5 and Fig 3.6. The proportion of cells actively proliferating in *Msx2*- or control-transfected cultures is lower than the basal proportion of actively proliferating cells. This suggests liposome-mediated transfection has a negative effect on PRE cell proliferation. Reduced proliferation in cells transfected with empty liposome vector has been observed (McPherson H,

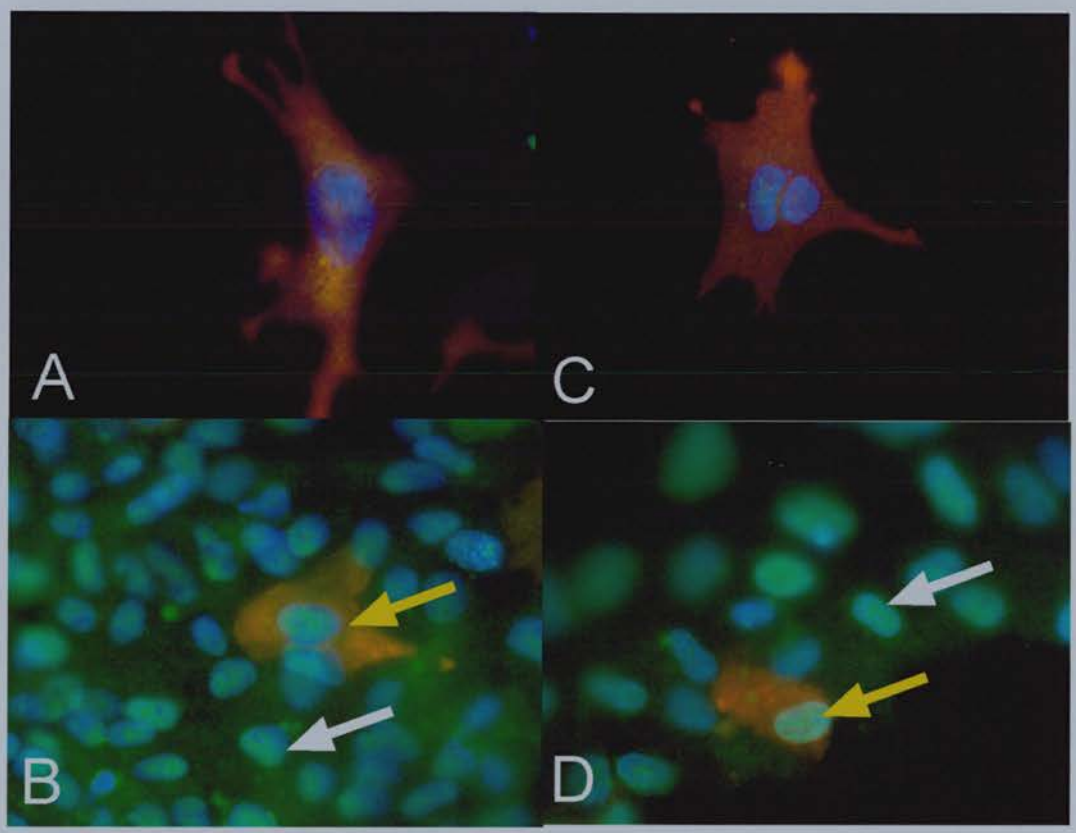


Fig3.7 A, Example of a m*Msx2*-transfected primary PRE cell, which is not expressing PCNA x100. B, Example of an untransfected PCNA-positive (white arrow) and a m*Msx2*-transfected PCNA-positive primary PRE cell (yellow arrow) x100. C Example of a control-transfected primary PRE cell, which is not expressing PCNA x100. D, Example of an untransfected PCNA-positive (white arrow) and a control-transfected PCNA-positive primary PRE cell (yellow arrow) x100.

personal communication) and this could be tested in PRE cells by including a control transfection with empty liposome vector. These experiments indicate that transfected PRE cells in the *in vitro* assay may have a reduced proliferative capacity.

Cell proliferation in primary PRE cells is not increased or decreased by ectopic expression of *Msx2*. These results fit with recent cell culture and *in vivo* data indicating that Msx1 does not promote cell proliferation but prevents cell cycle withdrawal by upregulating *cyclin D1* (Hu *et al.*, 2001). In cell culture Msx2 also upregulates *cyclin D1* (Hu *et al.*, 2001). Both Msx1 and Msx2 may maintain the proliferative state of cells by preventing cell cycle withdrawal, but not actively promoting or inhibiting cell division.

3.4 Identifying Msx candidate downstream genes by DIG in situ hybridization in the assay?

The key to understanding the functions of the Msx proteins during eye development is identification of their direct or indirect downstream target genes. The *in vitro* cellular assay can be used to investigate Msx downstream genes. A differential screen between material extracted from cells expressing or not expressing m*Msx2* could identify both known and unknown gene targets. Unfortunately, with the transfection efficiency of PRE cells in the *in vitro* assay I have calculated that the amount of mRNA which could be extracted from the *Msx2*-transfected cells would not be sufficient for a differential screen by either Representative Difference Analysis (RDA), Differential Display RT-PCR (DD-RT-PCR) or subtractive hybridization. An alternative is the candidate gene approach, where the expression of potential candidate *Msx* downstream genes are analyzed in *Msx2*- and control-transfected PRE cells.

Msx proteins appear to function directly as repressors of gene expression, although they may repress inhibitors and thus activate genes indirectly. Detecting gene repression, rather than activation, may be a greater challenge, since detecting a previously silenced transcript/protein is easier than detecting a decrease in expression level. Antibodies are available to the proteins coded for by a few Msx candidate genes, but for others only *in situ* probes or the gene sequence is available. An advantage of using antibodies to investigate candidate gene expression is that proteins are more readily detectable because they are generally more stable and more abundant than mRNA transcripts.

Msx-mediated repression may well involve small changes in mRNA level, so the method used for detecting mRNA needs to be relatively sensitive. The following experiments are a preliminary investigation into whether *in situ* hybridization using DIG-labelled probes could be used to investigate Msx2 downstream genes in the *in vitro* assay. In two independent experiments, duplicate cultures of dedifferentiated 5d chick PRE cells were transfected with the m*Msx2* and control expression constructs.

Following 48hrs in culture these cells were fixed and probed for m*Msx* mRNA expression using DIG-labelled m*Msx2* probes, table 3.6 and Fig 3.8.

Table 3.6 Number of m*Msx2* expressing cells detected using a DIG-labelled m*Msx2* probe on duplicate cultures of m*Msx2*-transfected dedifferentiated chick PRE cells in two independent experiments.

Experiment no.	Construct transfected	No. DIG – ve cells counted	No. DIG +ve cells	unknown	**% of total cells DIG +ve**
1	control	1627	0	10	0
		1942	0	15	0
	Msx2	3027	13	250	**0.43**
		3305	8	91	**0.24**
2	control	3145	0	0	0
		2956	0	0	0
	Msx2	4024	17	51	**0.42**
		5671	32	7	**0.564**

In control-transfected cultures no positive cells could be detected with DIG-labelled m*Msx2* probes, table 3.6 and Fig 3.7. In m*Msx2*-transfected cultures on average 0.41% (+/-0.23%) could be detected as m*Msx2*-expressing with DIG-labelled m*Msx2* probes, table 3.6 and Fig 3.8.

To estimate the number of m*Msx2*-transfected cells that DIG-labelled m*Msx2* probes can detect, the proportion of DIG-positive cells is divided by the transfection efficiency. The transfection efficiency for these experiments was estimated by counting the total number of cells and βGal-positive cells in five randomly selected fields of view in parallel cultures transfected with the *Msx2* and control construct at the same time and stained for βGal activity. However, it should be noted that the transfection efficiency in the cultures probed with the DIG-labelled probes was not

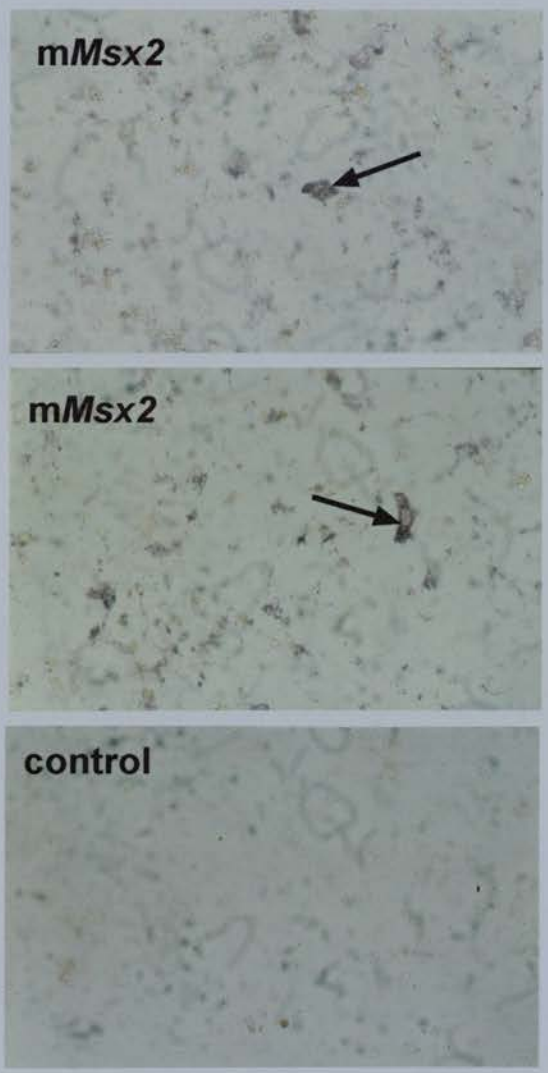


Fig 3.8 Detection of m*Msx2* mRNA in m*Msx2*-transfected cells using DIG-labelled m*Msx2* probes. Dedifferentiated PRE cells from 5d chick were transfected with either m*Msx2* or control construct, cultured for 48hrs, fixed and probed with m*Msx2*DIG-labelled probe. A, B, m*Msx2*DIG-positive cells in m*Msx2*-transfected cultures, x4. C, No m*Msx2*DIG-positive cells observed in control-transfected cultures, x4.

measured directly and may have been different from the cultures transfected in parallel, used to estimate the transfection efficiency.

The average transfection efficiency for experiment 1 was estimated to be 4.2% and the proportion of DIG-positive cells is 0.33%. Therefore, the estimated proportion of *Msx2*-transfected cells which are DIG-positive is 8%. In experiment 2 the average transfection efficiency was estimated to be 2.1% and the proportion of DIG-positive cells is 0.49%. This made the estimated proportion of *Msx2*-transfected cells which are DIG-positive is 23%. A DIG-labelled probe for m*Msx2* can thus detect a proportion of the PRE cells ectopically expressing m*Msx2*.

These results raise several questions; why m*Msx2* expression was detected in only a proportion of transfected cells? How sensitive are DIG-labelled probes and what expression level of transcript can they detect? Finally, could DIG-labelled probes be used to investigate Msx2 downstream genes?

The relative levels of βGal protein and DIG-labelled transcript required to produce detectable reactions may explain the difference in m*Msx2* expression and transfection efficiency. Within a population of transfected cells a range of protein and transcript expression levels will be present. Transfection was assayed by detection of the enzymatic activity of βGal. The amount of βGal protein required to produce a detectable reaction with X-Gal may be lower than the level of transcript required to produce a detectable reaction with a DIG-labelled probe. The expression level of *Msx2* and βGal transcripts should be the same. The levels of lacZ and m*Msx2* transcript in m*Msx2*-transfected cells and relative sensitivity of using DIG-labelled probes or βGal activity could be examined by *in situ* hybridization for lacZ and m*Msx2* expression using DIG-labelled probes and βGal activity on m*Msx2*-transfected cells. Alternatively, the amount of βGal protein may be increased relative to the number of *Msx2*/βGal transcript due to increased translational efficiency of the IRES. This could be examined by comparing expression of Msx and βGal proteins using respective antibodies.

So, how does the level of transcript detected in these experiments compare with expression levels of an endogenous gene and could this technique be used to investigate *Msx2* downstream genes? The *Msx2* expression construct uses the CMV promoter, in a transient transfection assay comparing the *CMV* promoter with the less active *yeast alcohol dehydrogenase* promoter (*pADH*), the *CMV* promoter has been found to be more than a thousand-fold active than the *pADH* (Lee *et al.*, 1998). In m*Msx2*-transfected PRE cells there may be a thousand-fold more transcripts of *mMsx2* than of any endogenously expressed transcript. The limits of detection of this technique could be tested by investigating expression of an endogenous PRE gene. An antibody to Mitf has shown that this protein is repressed in m*Msx2*-transfected PRE cells (Holme *et al.*, 2000). It would therefore be interesting to investigate *Mitf* mRNA levels in m*Msx2*-transfected and control-transfected cells by DIG *in situ* hybridization.

3.5 Investigating cellular functional redundancy between mMsx1 and mMsx2 with the in vitro assay

Proteins function at different levels; molecular, cellular and extracellular and they may be functionally redundant with other proteins at any of these levels. Msx1 and Msx2 share a highly conserved homeodomain and the molecular interactions with DNA and other proteins mediated via the homeodomain may be conserved between Msx1 and Msx2. Functional redundancy between proteins in cellular pathways may follow from conserved molecular functions, but could also result from different molecular interactions. Proteins may regulate different genes in a pathway or different genes in different pathways. The result of feedback within and crosstalk between cellular and extracellular pathways may be functional redundancy at the cellular and extracellular levels. In the vertebrate eye *Msx1* and *Msx2* are generally expressed in different tissues, yet they appear to have some functional redundancy during vertebrate eye development. To understand the functional redundancy of genes *in vivo* we need to examine functional redundancy at the cellular level. Do Msx1 and Msx2 have the same cellular functions and if they do are they mediated by conserved molecular interactions? Do Msx1 and Msx2 repress expression of the same genes, or different genes that have the same cellular effects? There is evidence from experiments in cell culture, where both Msx1 and Msx2 repress *cyclin D1,* that Msx1 and Msx2 are redundant at the level of cellular function (Hu *et al.*, 2001). The *in vitro* cellular assay provides a means to investigate the level at which functional redundancy may lie between Msx1 and Msx2.

In the work described so far in this chapter the assay for Msx2 cellular function is the formation of cells with a neural-like phenotype. The small number of *Msx2*-transfected cells which display the dendritic phenotype is a severe limitation on further use of the assay to investigate Msx cellular function. However, *Msx2*-transfection has been found to repress expression of the key PRE protein Mitf in a large number of *Msx2*-transfected cells. Expression of Mitf was found to be downregulated in 52% of *Msx2*-transfected cells, in comparison with control-transfected PRE cells (Holme *et al.*, 2000). Furthermore, in the mouse eye, *Msx2* is expressed in the prospective NR, where *Mitf* is expressed initially, but is later

downregulated. *In vivo* Msx2 may downregulate *Mitf* in the prospective NR cells of the optic vesicle. Using the downregulation of Mitf as an assay for Msx2 cellular function increases the number of cells available in which to further investigate the cellular functions of Msx2.

In chick PRE cells two cellular effects of *Msx2* expression are observed, the formation of dendritic cells and downregulation of expression of the key transcription factor Mitf. Initially, the cellular functional redundancy between Msx1 with Msx2 was investigated using the formation of cell with a neural-like phenotype as an assay.

3.5.1 Dendritic cells

In two independent experiments 5d chick dedifferentiated PRE cells were transfected with either the m*Msx1*, m*Msx2* or control construct, (see section 2.2.4 in Chapter 2 Materials and Methods and Appendix1), and cultured in serum-free media. 48 hrs after transfection the cells were fixed and stained for β-Galactosidase activity and assayed for dendritic cell formation, table 3.7 and Figs 3.9 and 3.10.

Table 3.7 Proportion of *Msx1*- and control-transfected dedifferentiated PRE cells in two independent experiments displaying a dendritic phenotype in serum-free culture conditions. The number of transfected cells counted represents an estimated 80% of those transfected

	Construct transfected					
	Msx1		*Msx2*		control	
	Experiment no.					
	1	2	1	2	1	2
Number of transfected cells	834	582	991	1058	525	640
Number with a dendritic morphology	15	2	21	3	0	0
% of transfected cells with a dendritic morphology	1.8	0.34	2.1	0.28	0	0

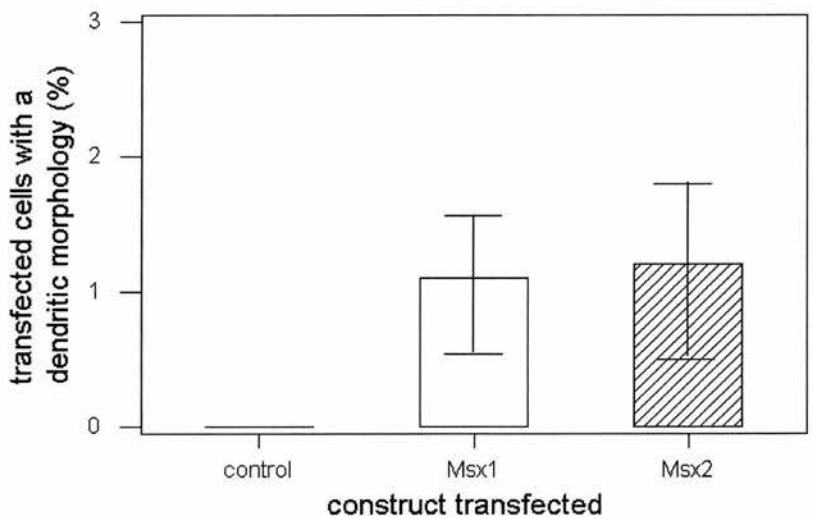


Fig. 3.9. Proportion of *mMsx1*- and control-transfected dedifferentiated PRE cells in two independent experiments with a dendritic morphology.

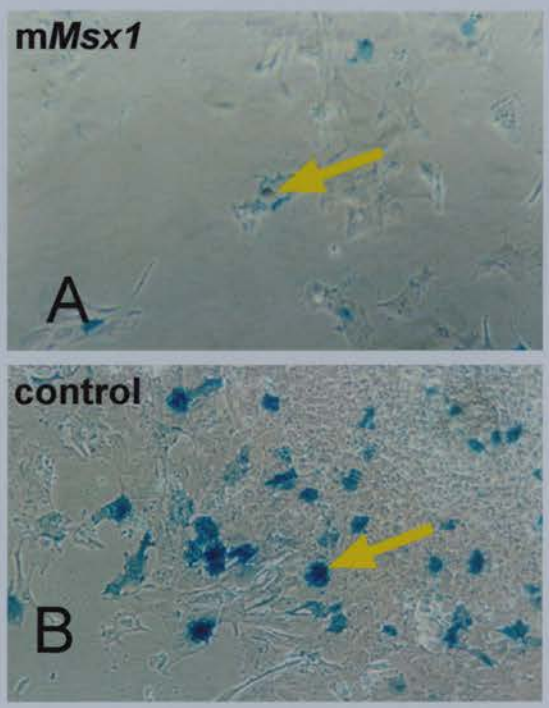


Fig. 3.10 Formation of cells with a dendritic morphology in m*Msx1*-transfected PRE cells. Dedifferentiated PRE cells from 5d chick were transfected with either m*Msx1* or control construct, cultured for 48hrs, fixed and stained for Bgal. A, a m*Msx1*-transfected cell with a dendritic morphology, x4. B, control-transfected cells with regular PRE cell morphology, x4.

A small number of m*Msx1*- and *Msx2*-transfected cells had a dendritic phenotype and no dendritic cells were observed in the control-transfected cells, table 3.7, Figs 3.9 and 3.10. In common with *Msx2*, m*Msx1* can promote the formation of dendritic cells in chick PRE cells. These results were subsequently confirmed in primary PRE cells (Holme, 1998). The proportion of transfected cells with a dendritic phenotype in *Msx1*- and *Msx2*-transfected cultures shows significant variation between experiments. To examine the variation in the proportion of *mMsx2*-transfected dedifferentiated cells developing a dendritic phenotype the data from three independent experiments were pooled, table 3.8 and Fig 3.11.

Table 3.8, Summary of number of cells with a dendritic phenotype in three experiments using dedifferentiated PRE cells.

Experiment no.	Construct transfected	No. of transfected cells	No. with dendritic morphology	% with dendritic morphology
1	*Msx2*	2030	93	4.58
	control	2192	0	0
2	*Msx2*	991	21	2.1
	control	525	0	0
3	*Msx2*	1058	3	0.28
	control	640	0	0

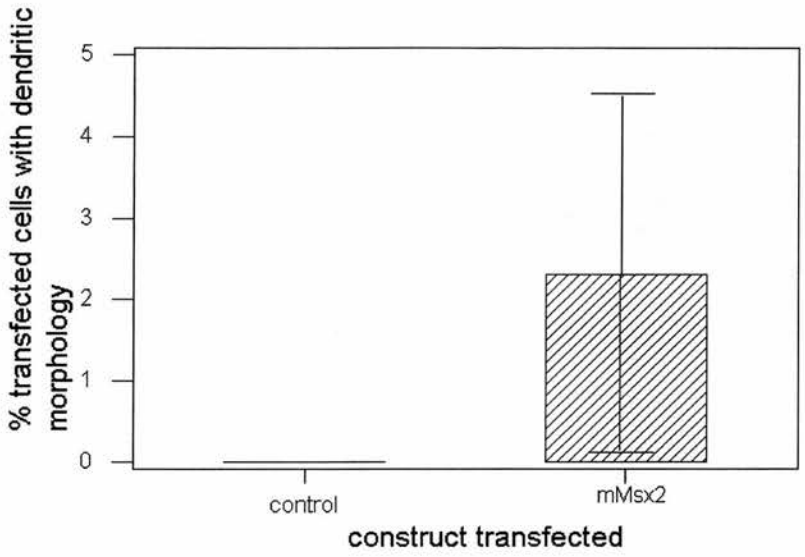


Fig 3.11. Graph showing the percentage of transfected dedifferentiated chick PRE cells with a dendritic morphology in three independent experiments

Table 3.8 and Fig 3.11 show the average proportion of m*Msx2*-transfected dedifferentiated PRE cells with a dendritic phenotype is 2.32 (+/- 2.16%). There is a high degree of variation in the proportion of m*Msx2*- transfected cells with a dendritic phenotype. Prior to transfection all these cells were passaged 5 times so the number of passages cannot account for the variation. These results suggests that additional factors, for example; cell density or growth factors released by the PRE cells themselves may be contributing to the formation of the dendritic phenotype. Therefore, until the source of this inherent variation in the assay is established it is not possible to draw meaningful conclusions from the proportion of m*Msx2*-transfected dedifferentiated PRE cells with a dendritic phenotype. The effect of cell density on formation of dendritic cells could be investigated by plating out and transfecting dedifferentiated PRE cells at different densities and counting the number of dendritic cells in the different cultures. This variation, in addition to the low proportion of m*Msx2*-transfected cells with a dendritic phenotype, is another reason

why the formation of dendritic cells is not an ideal assay for investigating Msx cellular function.

3.5.2 Mitf downregulation

The downregulation of Mitf is a cellular function of *Msx2* in PRE cells in culture. Examining the expression of Mitf in *Msx1*-transfected PRE cells may reveal whether the two genes are functionally redundant in their effect on Mitf. However, further experiments will be required to understand the nature of any functional redundancy between *Msx1* and *Msx2*. In two independent experiments dissociated 5d embryonic primary chick PRE was transfected with m*Msx1* or control construct and Mitf expression in transfected cells was analyzed. After 48 hrs in culture the cells were fixed and mouse anti-βgalactosidase and rabbit anti-Mitf antibodies applied and visualized using the corresponding secondary anti-bodies; anti-mouse FITC and anti-rabbit Texas Red. βgal expressing cells were scored for Mitf expression, tables 3.9 and 3.10 and Figs 3.12 and 3.13. The proportion of βGal-positive cells which are *Msx1*-positive is not known but this could be investigated using antibodies to βGal and Msx on *Msx1*-transfected PRE cell cultures.

Table 3.9 Mitf expression in *Msx1*- and control transfected 5d primary PRE cells. The number of transfected cells represents an estimated 50% of the total number of cells transfected.

	Experiment no.			
	1		2	
	Construct transfected			
	Msx1	control	*Msx1*	control
Number of cells βgal+ve/Mitf-ve	38	14	26	9
Number of cells βgal+ve/Mitf+ve	22	64	36	47
Number of cells βgal+ve/Mitf unknown	7	24	10	10
Total number of cells	67	102	72	66

Table 3.10 Proportion of *Msx1*- and control transfected 5d primary PRE cells expressing Mitf.

	Experiment no.					
	1		2		**Mean**	
	Construct transfected					
	Msx1	control	*Msx1*	control	***Msx1***	**control**
% βgal+ve/Mitf-ve	57	14	36	14	46.5	14
% βgal+ve/Mitf+ve	33	63	50	71	41.5	67
% βgal+ve/Mitf unknown	10	23	14	15	12	19

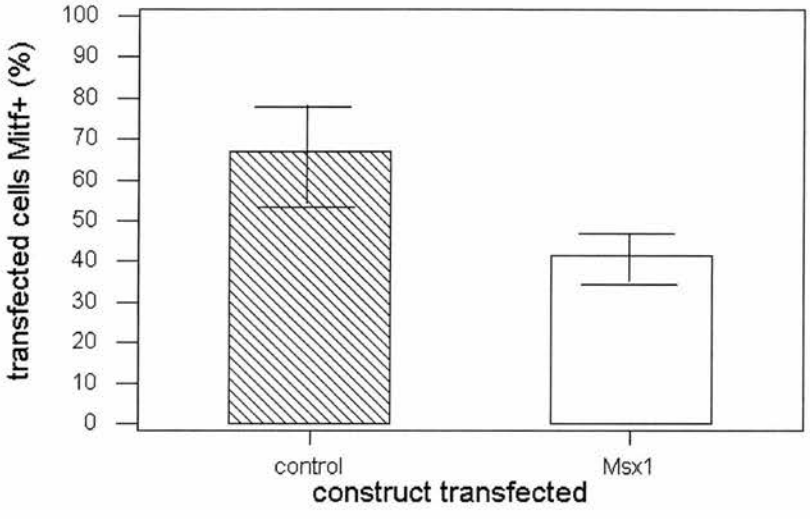


Fig.3.12. Percentage of untransfected, m*Msx2*- and control-transfected cells Mitf+. Cells classed as unknown were considered equally likely to be Mitf+ or Mitf- and are not included in the bar chart.

Table 3.10 and Fig 3.12 show that an average of 41.5% (+/-12%) of m*Msx1*-transfected cells, compared with 67% (+/-5.6%) of control-transfected cells express Mitf. The chi-squared test indicates that the difference in Mitf expression between m*Msx1*- and control-transfected cells is statistically significant (χ^2= 35.459, d.f.=1, p=<0.01). Comparing m*Msx1*- transfected with control-transfected PRE cells 24 % of *Msx1*- transfected cells do not express the PRE marker Mitf. Thus, ectopic expression of *Msx1* in PRE cells, in common with m*Msx2* (Holme *et al.*, 2000), downregulates expression of Mitf in PRE cells in a cell-autonomous manner. Thus, in the *in vitro* assay m*Msx1* shows functional similarity with m*Msx2* in the cellular functions of promoting dendritic cell formation and downregulating Mitf expression.

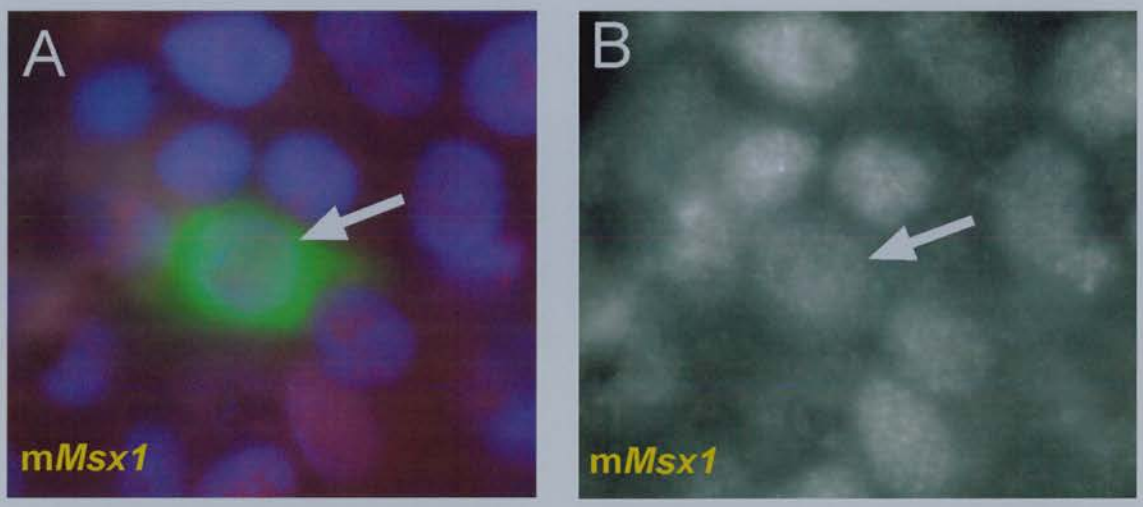


Fig3.13 A; Example of an m*Msx1* transfected and MITF-positiveprimary PRE cell x100. Anti-rabbit BGal and anti-mouse MITF primary antibodies were used with anti-rabbit FITC (green) and anti-mouse Texas Red (red) secondary antibodies. B; grey level image of the m*Msx1*-transfected cell in A positive with the anti-mouse MITF primary and anti-mouse Texas Red x100.

During mouse eye development *Msx2* is expressed in the prospective neural retina domain of the optic vesicle where *Mitf* is expressed initially but then becomes downregulated, (Holme, 1998; Monaghan *et al.*, 1991; Nguyen and Arnheiter, 2000). Downregulation of *Mitf* by ectopic *Msx2* in the *in vitro* assay may represent an *in vivo* cellular function of Msx2. To investigate whether the regulation of *Mitf* represents an *in vivo* cellular function of Msx2, *Mitf* expression in the *Msx2*-null mutant mice could be analyzed. However, a lack of effect could be due to functional redundancy of Msx2 with another protein. Molecular interactions conserved between Msx1 and Msx2 may be mediating *Mitf* downregulation in the *in vitro* assay.

There are a number of possible mechanisms by which ectopic *Msx1* and *Msx2* expression in the *in vitro* assay may be promoting downregulation of *Mitf* expression. First, Msx1 and Msx2 may bind directly to *Mitf* regulatory region and repress *Mitf* transcription. *In vitro* Msx1 binds to the core enhancer region of the key bHLH transcription factor *MyoD,* which has a similar role to Mitf in muscle cells (Woloshin *et al.*, 1995).

Secondly, Msx1 and Msx2 may downregulate *Mitf* by specific interference with the activity of homeodomain transcription factors. Muscle precursor cells migrating to the limb appear to co-express *Msx1* and Pax3 (Bober *et al.*, 1994; Houzelstein *et al.*, 1999; MacKenzie *et al.*, 1997). There is *in vitro* evidence Msx1 forms a heterodimer with Pax3 which blocks the activating action of Pax3 on the MyoD promoter (Bendall *et al.*, 1999). Lastly, overexpression of *Msx1* and *Msx2* from the CMV promoter and subsequent high levels of protein may be resulting in general gene repression by interference with the transcription machinery.

A number of experiments could be designed to address which of the above mechanisms is in operation. The same regions of the Msx homeodomain are – involved in DNA-binding and protein-protein interactions. Helix III lies in the major groove of the double helix and the N-terminal arm lies in the minor groove, both make contact with the DNA. *In vitro* binding assays with amino acid substitutions in

the N-terminal arm, Helix I, II and III of the Msx1 homeodomain, suggest that the N-terminal arm and Helix III are essential for both DNA-binding and interactions with Pax3, Lhx2 and Dlx2 and 5 (Bendall *et al.*, 1998a; Zhang *et al.*, 1997). The N-terminal arm of the homeodomain of Msx1 and Msx2 also mediates interactions with components of the transcription machinery which in co-transfection assays are required for *in vitro* repression (Newberry *et al.*, 1997; Zhang *et al.*, 1996). This makes it difficult to design amino acid substitutions in the Msx homeodomain that could distinguish between repression involving direct DNA-binding by Msx2 and specific interference of homeodomain proteins by Msx2 binding.

One way to investigate the downregulation of *Mitf* by *Msx1* and *Msx2* is to analyze the timing of Mitf downregulation, by immunohistochemistry at different time points after transfection with the *Msx2* and control constructs. This may give an indication of how direct the relationship between *Mitf* downregulation is to *Msx1* and *Msx2* expression. As a means to investigate a direct interaction between *Msx1*, *Msx2* and the *Mitf* promoter dominant positive and dominant negative fusion proteins could be produced. The Msx homeodomain could be fused to the VP16 activation domain (Friedman *et al.*, 1988) or the repressor domain of Engrailed (En^r) (Badiani *et al.*, 1994; Bao *et al.*, 1999; Conlon *et al.*, 1996; Furukawa *et al.*, 1997; Yu *et al.*, 2001). The effect on Mitf expression could be examined in *Msx*Vp16-, *Msx* En^r - and control-transfected PRE cells.

Finally, the cellular effects observed in the *in vitro* assay may be specific to Msx1 and Msx2 or they may be due to non-specific interference as a result of high expression of a homeodomain protein. To investigate whether the effects are specific to Msx1 and Msx2 a similar homeobox repressor protein, for example engrailed could be expressed in primary PRE cells, under control of the CMV promoter. The formation of dendritic cells and Mitf expression in engrailed- and control- transfected cells analyzed.

Summary and conclusions

In this chapter I have investigated the practical application of an *in vitro* assay for studying Msx cellular functions and downstream genes. Ectopic expression of *Msx2* in dedifferentiated PRE cells promotes the formation of cells with a neural-like morphology, as observed originally in primary PRE cells. Using dedifferentiated PRE cells in the assay substantially increases the number of cells available in which to investigate the cellular functions of the Msx proteins. The formation of cells with the neural-like morphology is not dependent on serum growth factors and the proportion of *Msx2*-transfected cells developing a neural-like phenotype is not markedly increased by neural-specific culture conditions. I have found no evidence of an increase or decrease in PRE cell proliferation as a result of ectopic *Msx2* expression. DIG-labelled probes can detect ectopic m*Msx2* expression in *Msx2*-transfected PRE cells. Using the *in vitro* assay to investigate cellular functional redundancy I discovered that ectopic *Msx1* in primary PRE cells also promotes a small number of *Msx1*-transfected cells to develop a neural-like phenotype. Furthermore, *Msx1*-transfection results in Mitf downregulation in a significant proportion of PRE cells. Suggesting that, at least in these cellular functions, Msx1 and Msx2 are functionally redundant. To answer the key question; whether the *in vitro* cellular assay represents *in vivo* cellular functions of mMsx1 and mMsx2, the next chapter describes my attempts to ectopically express *Msx2* in the mouse PRE *in vivo*.

Chapter 4 Ectopic expression of *Msx2* in the PRE of transgenic mice

4.1 Introduction

In the *in vitro* cellular assay ectopic expression of mouse *Msx2*, by transient transfection in cultures of chick PRE cells, promotes neural characteristics in a small number of transfected cells and causes downregulation of the key PRE transcription factor, Mitf, in a large proportion of transfected cells. Both Mitf downregulation and development of neural characteristics suggest *Msx2* may have functions in neural specification or PRE fate suppression in optic vesicle cells. These are consistent with the expression of *Msx2* in the prospective neural retinal domain of the mouse optic vesicle. However, the *in vitro* assay is based on the function of mouse *Msx2* in chick PRE cells. There may be subtle cellular differences between chick and mouse PRE cells and differences in the structure of mouse and chick Msx2 may result in ectopic mouse *Msx2* having a cellular effect in chick PRE that does not precisely mimic its role in mouse PRE. In addition, to possible species-specific differences, PRE cells *in vivo* are surrounded by their normal cellular environment and maintain their normal cell-cell and cell-ECM contacts which they do not have in culture. It is therefore essential to test whether the cellular effects observed when *Msx2* is expressed ectopically in PRE cells in culture represent *in vivo* cellular functions of the Msx proteins. We adopted the *in vivo* strategy of generating transgenic mice to ectopically express *Msx2* in the PRE during normal retina development under control of the *Trp1* or *Trp2* promoters.

As described previously, *Msx2* is expressed during mouse eye development in the surface ectoderm and the presumptive NR domain, but not the PRE, of both the distal optic vesicle and later the optic cup. When both PRE and NR domains are differentiating *Msx2* expression is maintained in a small area of the presumptive ciliary body region. *Trp1* and *Trp2* code for proteins with roles during the synthesis of pigment within the PRE. Tyrosinase related protein 2 (Trp2) converts DOPAchrome into 5,6-dihydroxyindole 2-carboxylic acid (DHICA), (Mackenzie *et*

al., 1997), whilst tyrosinase related protein 1 (Trp1) converts DHICA into 5, 6-quinone 2-carboxlic acid (Kobayashi *et al.*, 1994). In the mouse, *Trp1* is expressed in the PRE from E11.5, onwards and *Trp2* is first seen at E9.5 in the prospective PRE domain of the optic vesicle, (Steel *et al.*, 1992). Mice carrying the brown mutation, which are null for *Trp1*, (Shibahara *et al.*, 1991, Jackson, personal communication) have normal, pigmented eyes indicating that this gene is not essential for eye pigmentation. The eyes of mice with a mutation in *Trp2* are also pigmented; however, it is not known if this is a null mutation and consequently whether TRP2 is essential for the production of pigment in the eye (Budd and Jackson, 1995). 1.4kb of *Trp1* promoter, extending from –1334 to 107 and containing part of the first exon, directs LacZ expression to the PRE from E11 onwards and has been used to successfully ectopically express diphtheria toxin-A in mouse PRE cells (Raymond and Jackson, 1995). 3.6kb of *Trp2* promoter, extending from 454 to -3181, directs LacZ expression to the presumptive PRE from late E9.5 onwards (Mackenzie *et al.*, 1997). From these experiments the promoters of *Trp1* and *Trp2* appeared to be good candidates for driving ectopic *Msx2* expression in mouse PRE cells.

Previous work showed the *Trp1* promoter actively drives *mMsx2* expression when transiently transfected into chick PRE cells, but no transgene activity could be detected in stable transfected mouse lines. A construct was produced driving *Msx2* expression from 1.8kb of the *Trp1* promoter. To monitor expression of the transgene, an IRES/βGeo cassette was cloned downstream of the m*Msx2* cDNA, to produce βGal in cells where *mMsx2* is ectopically expressed. Translation of βGal in transgenic mouse embryos, mediated by IRES sequences has been described (Kim *et al.*, 1992; Mountford *et al.*, 1994). Expression of the construct was first checked in chick PRE cells in culture by RT-PCR for *mMsx2* and by staining for βGal activity. RT-PCR with primers for mouse *Msx2* on RNA isolated from *Trp1*m*Msx2*IβGeoSV40-transfected PRE cells amplified the predicted sized transgenic fragment and which was absent when RNA from control-transfected PRE was used. A large number of cells in *Trp1*m*Msx2*IβGeoSV40-transfected cultures showed βGal activity (Holme, 1998). No cells with dendritic morphology were observed in chick PRE cells transfected with the control construct but a single cell

with apparent dendritic morphology were observed in *Trp1*m*Msx2*IβGeoSV40-transfected PRE cells (total cells counted 1500). These results indicate that in chick PRE the *Trp1* promoter was active and efficiently produces βGal (Holme, 1998). Four stable transgenic lines were made by DNA micro-injection of the *Trp1*m*Msx2*IβGeoSV40 transgene. However, all the embryos shown to be transmitting the transgene by PCR had normal eye morphology and no βGal staining at any of the stages looked at (between E10.5 and E15.5), (Holme, 1998). The reasons for this lack of transgene activity are discussed later in this chapter.

A transgene construct was then produced with 3.6kb of the previously defined *Trp2* promoter driving expression of m*Msx2* and including the IRES/βGeo reporter gene cassette. To confirm the activity of the p*Trp2Msx2*IβGeoSV40 construct it was transiently transfected into chick PRE cells in culture. A large proportion of cells in the p*Trp2Msx2*IβGeoSV40 transfected cultures showed βGal activity confirming that this construct efficiently produces βGal in chick PRE cells in culture but no transfected cells were observed with a dendritic morphology (Holme, 1998). The *mMsx2* cDNA in the construct was partially sequenced to check its integrity and orientation and this confirmed the orientation of the *mMsx2* insert. Two base changes from the published sequence were found in the 5' untranslated region on the anti-sense strand but since they were 5' of the translation start site they were not expected to affect the structure of Msx2 protein (Holme, 1998).

Micro-injection of the p*Trp2Msx2*IβGeoSV40 construct produced four transgenic male founders; A80.1, A81, A81.3 and A88. 50% of the embryos from A80.1, A81.3 and A88 were transgenic judged by PCR analysis of DNA extracted from embryonic yolk sacs. Between 16 and 26 whole embryos from the lines A80.1, A81.3 and A88, (at ages ranging from E9.5 to 13.5), were fixed and stained for βgal. None showed any βgal activity and all had gross eye morphology indistinguishable from wild type siblings (Holme, 1998). In transgenic embryos from line A81 small patches of βGal staining were observed in the PRE of transgenic embryos at all stages analysed (E9.5, E10.5, E11.5 and E13.5), (Holme, 1998). In whole mounts and sections the

gross morphology and degree of pigmentation of A81 transgenic E9.5, E11.5 and E13.5 eyes was indistinguishable from wild type siblings, (Holme, 1998).

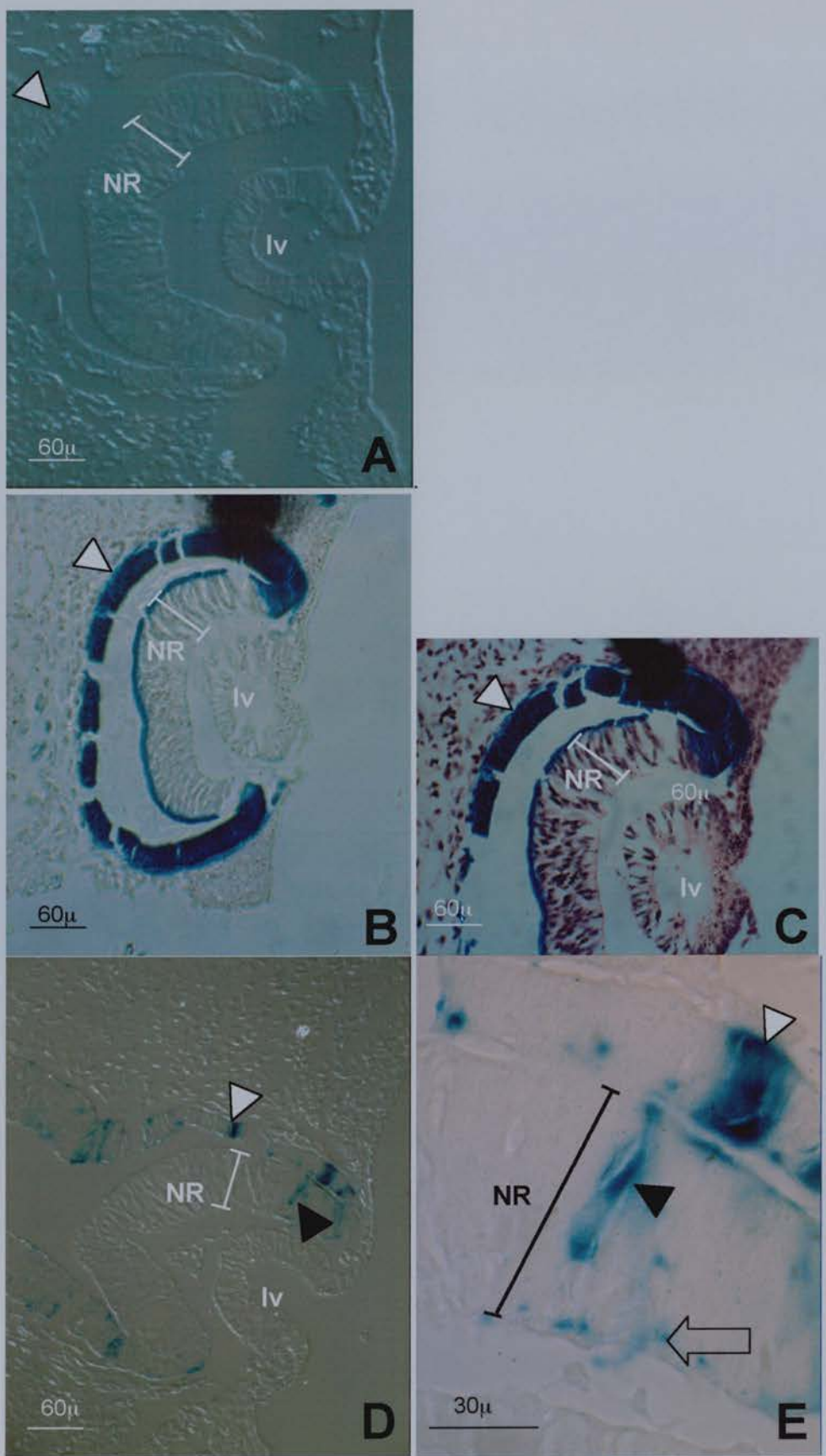
4.2 Analysis of A81 mice

4.2.1 Patches of βgal expression are observed in the eyes of the transgenic line A81

The small patches of βGal suggested that the transgene was active in some of the PRE cells in the mice of line A81 (A, C, D Fig 4.1). Furthermore, in some sections through a few eyes of E11.5 and E10.5 transgenic A81 mice a small number of βGal-positive cells are observed in the inner layer of the presumptive ciliary margin (D and E, Fig 4.2).

Previously, five out of five stable transgenic lines expressing LacZ under control of the 3.6kb of *Trp2* promoter showed βGal expression in the PRE from E9.5 to E12.5 (B, Fig 4.1), (Mackenzie *et al.*, 1997), but the domain of expression in the retina had not been studied in detail. The eyes of E10.5 mice from one of these lines, A12, were therefore sectioned (B and C, Fig 4.2) to compare the expression level and pattern with that of the A81 mice, (D and E, Fig 4.4). The expression domain of βGal in the A12 mice extends into the presumptive ciliary margin stopping where the presumptive neural retina begins, (B, C, Fig 4.2). In comparison, in the A81 mice βGal is only expressed in small patches of PRE cells (D, Fig 4.2). Punctate spots of localised βGal are seen in the PRE and inner layer of the presumptive ciliary margin of the A81 mice (E, Fig 4.2) characteristic of low Gal activity (Murphy *et al.*, 1996). The low level and patchy expression pattern of βGal in the A81 mice may be a result of poor translation from the IRES sequence. A small number of βGal-positive cells in the presumptive neural retina are outside the normal domain of *Trp2* expression (B, D, Fig 4.2) and there are several possible explanations for this ectopic βGal expression. Ectopic expression of the *Msx2* transgene in the PRE may be affecting the development of some cells, potentially promoting their differentiation along a neural pathway. Alternatively, the transgene may have been expressed in a small

number of cells at the border between the PRE and NR which are fated to form part of the neural retina. The transgene may no longer be active in these cells but the βGal protein is detectable in these cells because of it is stable for 24hrs. Finally, transgene position effects may produce subtle differences between expression of the transgene and native *Trp2* resulting in ectopic expression of the transgene in some cells of the neural retina cells. To investigate whether the transgene was actively expressing *Msx2* ectopically in the PRE of the A81 mice expression of *Msx2* was analyzed by *in situ* hybridization.


NR
lv
60µ
A
NR
lv
60µ
B
NR
60µ
lv
60µ
C
NR
lv
60µ
D
NR
30µ
E

4.2.2 Ectopic expression of *Msx2* could not be detected in the PRE of E10.5 or E11.5 embryos from line A81

Sections of wax-embedded embryos of wildtype and transgenic A81 mice (E10.5d and E11.5d) were hybridized *in situ* with S^{35} radiolabelled *mMsx2* RNA probe. Native *Msx2* expression was observed in the surface ectoderm of the head, in the lens vesicle (at E10.5d, see Figs 4.3 and 4.4) and in the proximal NR at E11.5d (see Fig. 4.5) and surface ectoderm at E11.5d (see Figs 4.5 and 4.6) . No ectopic *Msx2* mRNA expression was detected at E10.5d or E11.5d in the PRE of transgenic A81 mice (see Figs 4.3 and 4.5). There is no evidence for *Msx2* transgene activity at E10.5 or E11.5, in the PRE of A81 mice. Either the level of transgene expression is beyond the limits of detection by *in situ* hybridization or the transgene is silenced in the majority of the cells in the PRE of A81 mice.

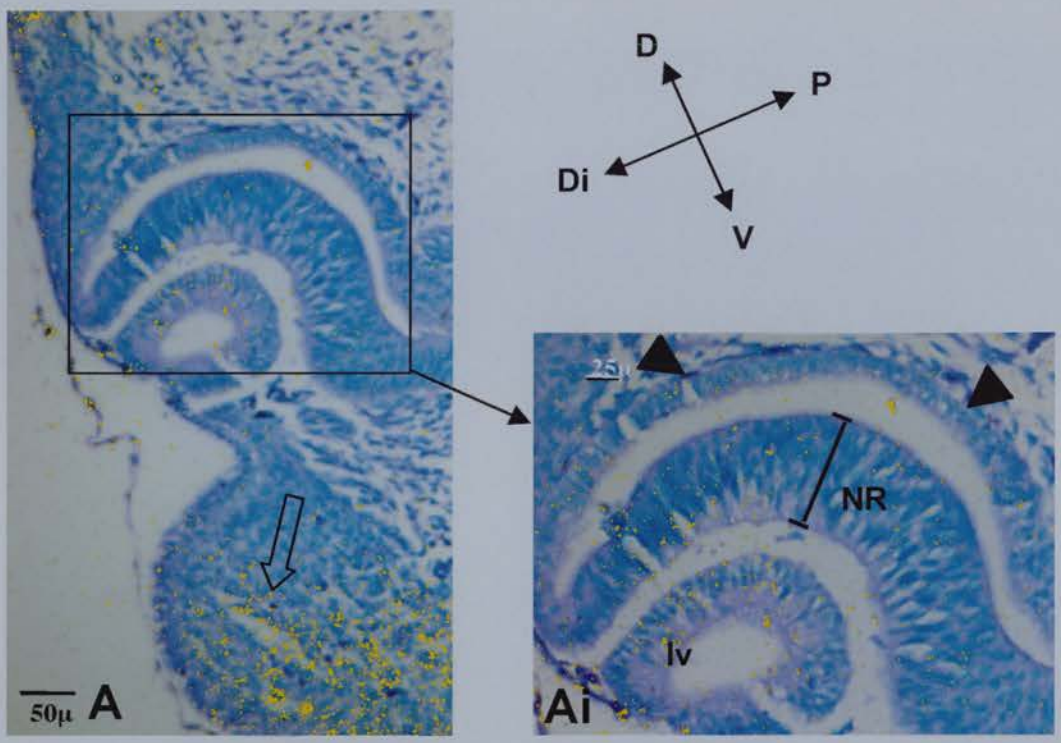


Fig 4.3 *Msx2* expression in eye region of E10.5 mouse from the transgenic line A81which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the surface ectoderm and mesenchyme ventral to the eye, (open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

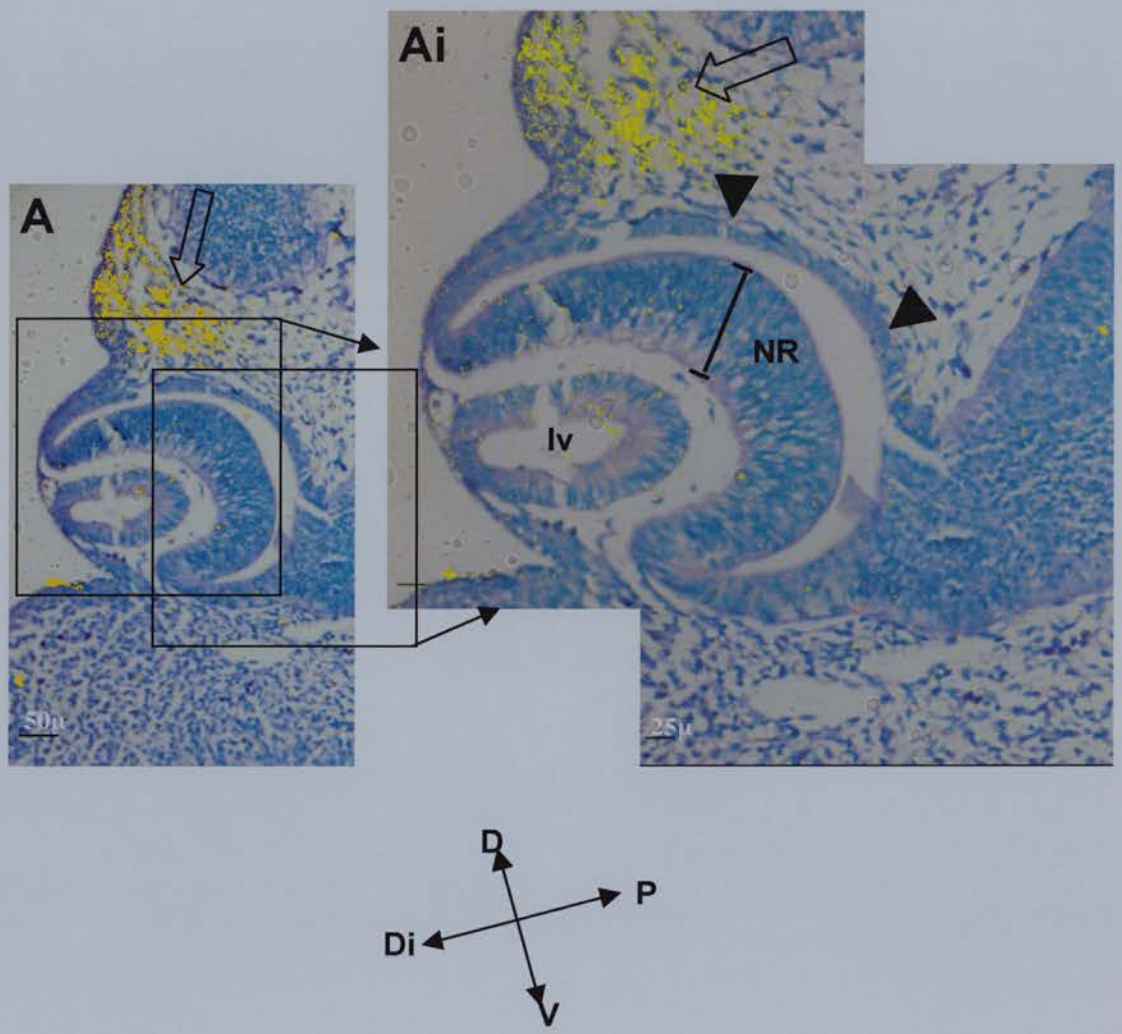


Fig 4.4 *Msx2* expression in eye region of E10.5 wildtype mouse A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the surface ectoderm and mesenchyme dorsal to the eye, (open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

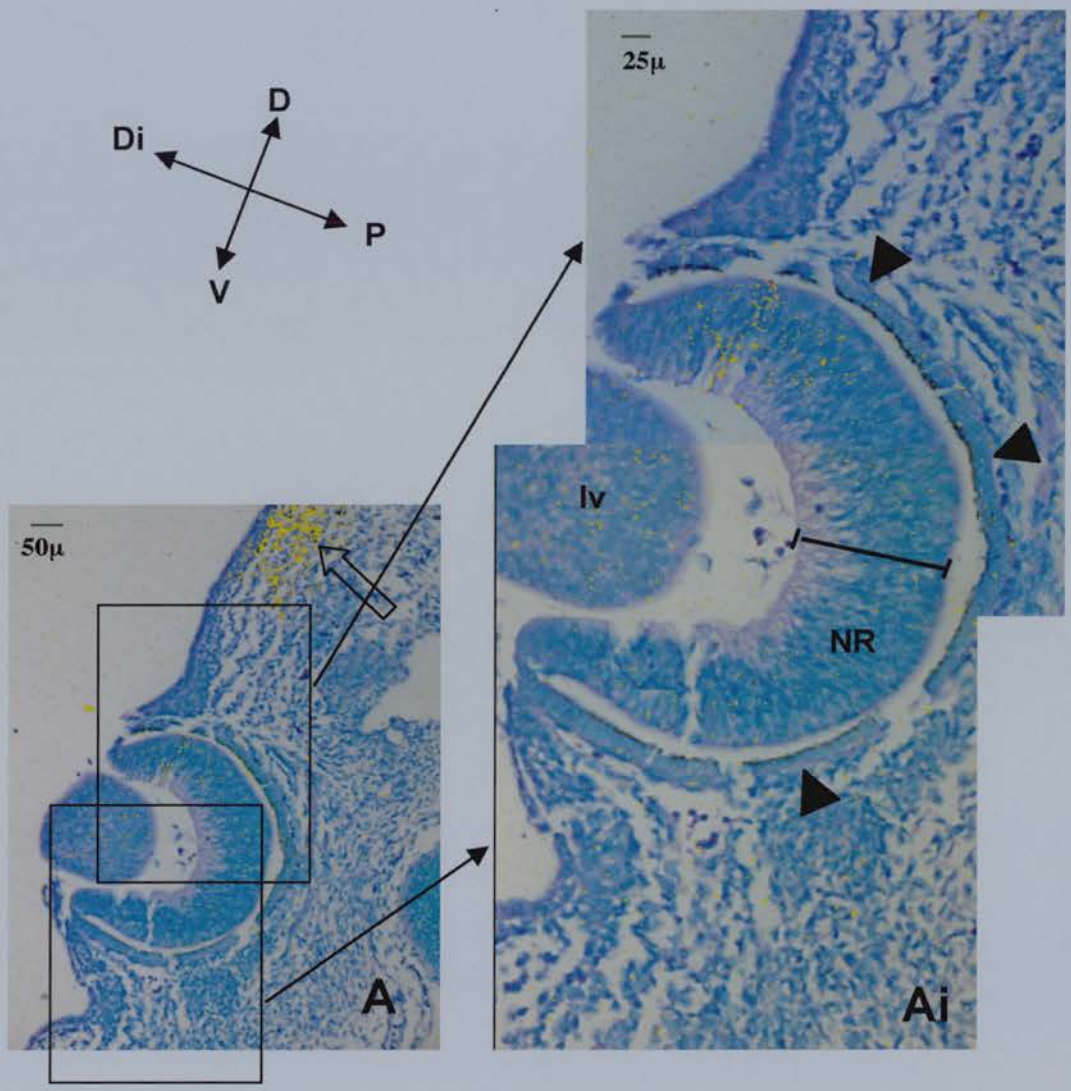


Fig 4.5 *Msx2* expression in eye region of E11.5 mouse from the transgenic line A81which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the mesenchyme dorsal to the eye, (open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

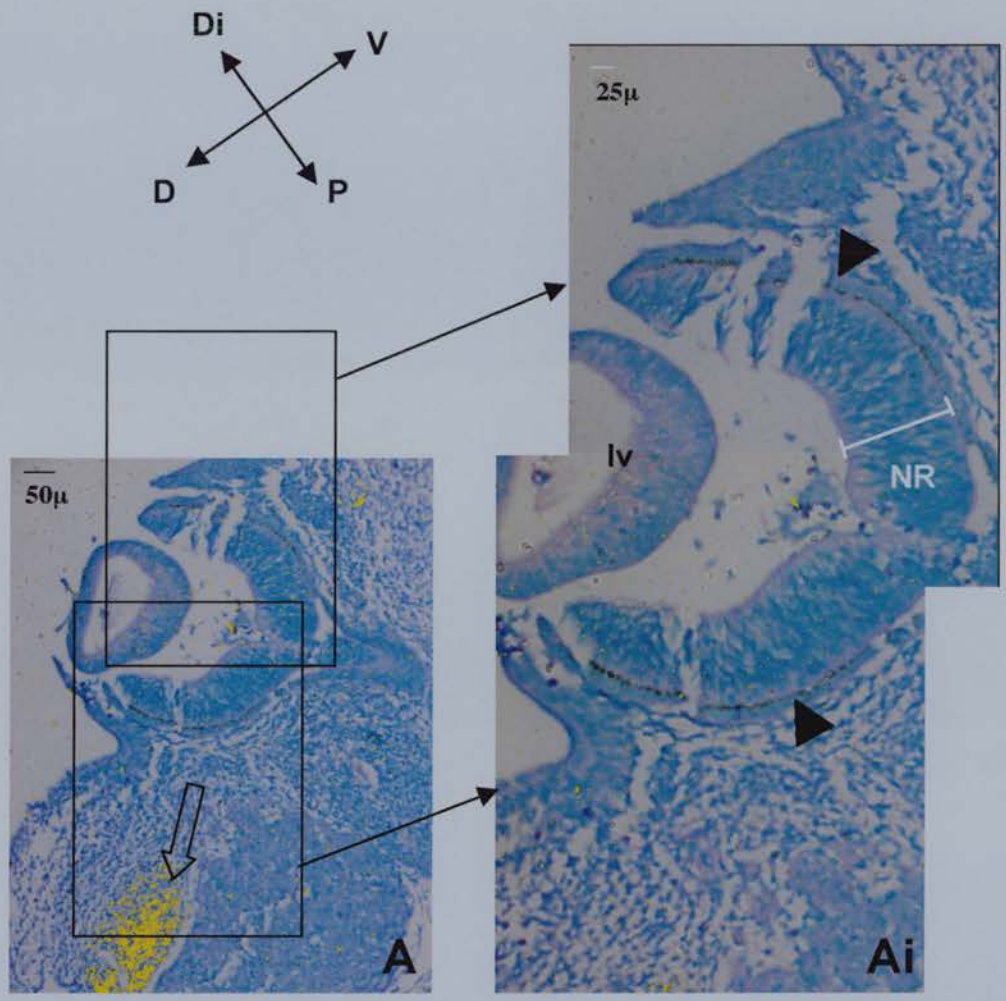


Fig 4.6 *Msx2* expression in eye region of E11.5 wildtype mouse A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the mesenchyme dorsal to the eye, (open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

4.2.3 *Trp2* expression is normal in the eyes of transgenic embryos from line A81

One possibility is that *Msx2* may repress *Trp2* expression. Expression of *Msx2* from the transgene may therefore repress transgene activity by a autorepressive mechanism. There is no evidence for *Trp2* repression by *Msx2* but this could be investigated in the *in vitro* assay by immunohistochemistry for *Trp2* expression in m*Msx2*-transfected chick PRE cells.

To investigate whether ectopic expression of *Msx2* may be downregulating *Trp2* and silencing transgene activity *in vivo* in the PRE of the A81 embryos the expression of endogenous *Trp2* was analyzed by *in situ* hybridization in these mice. Sections of wax-embedded embryos of wildtype and transgenic A81 mice (E10.5d and E11.5d) were hybridized with a S^{35} radiolabelled *Trp2* RNA probe. Expression of *Trp2* mRNA is observed in the outer layer of the optic cup, the presumptive PRE, at E10.5 and E11.5, in both A81 (see Figs 4.7 and 4.9) and wildtype embryos (Figs 4.8 and 4.10). The expression level and distribution of *Trp2* mRNA in the PRE of A81 transgenic embryos compared and wildtype embryos (Figs, 4.7, 4.8 and 4.9, 4.10) shows no significant difference. Expression of *Trp2* in transgenic embryos from line A81 appears normal. Autorepression of the *Msx2* transgene as a result of *Trp2* repression by ectopic *Msx2* does not appear to be the reason for the lack of detectable transgene activity in the A81 mice.

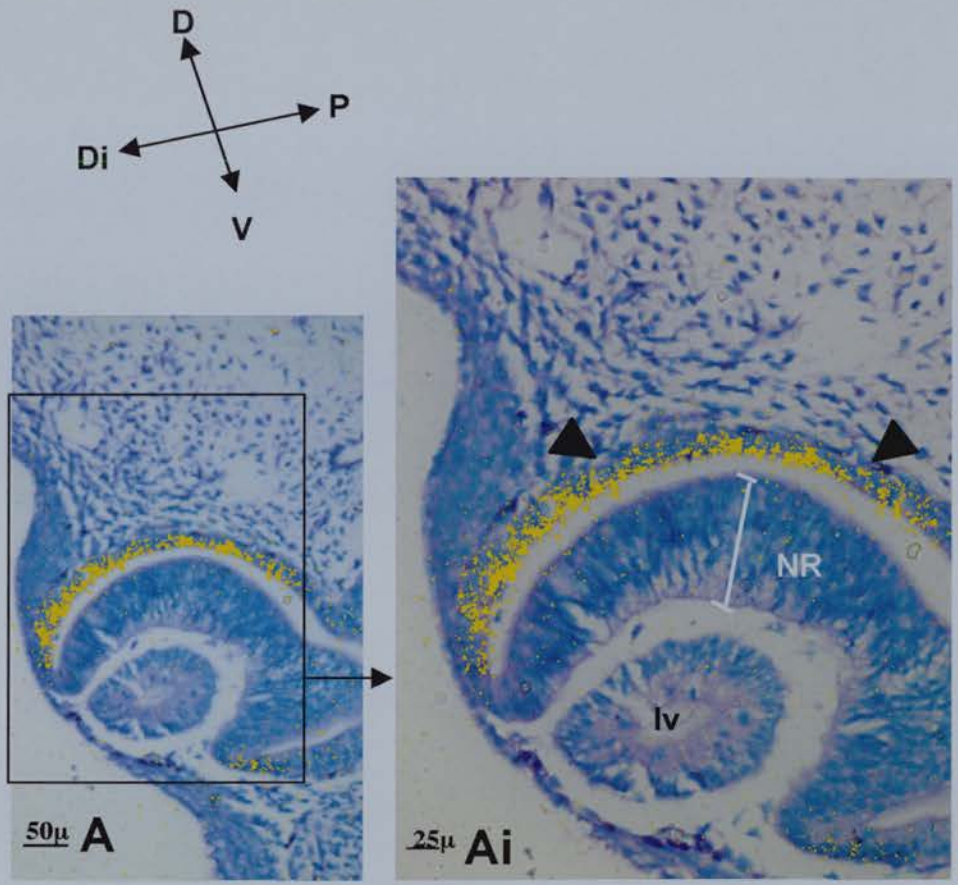


Fig 4.7 *Trp2* expression in eye region of E10.5 mouse from the transgenic line A81 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). Ai) enlargement of the retina. No *Trp2* expression was observed in NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

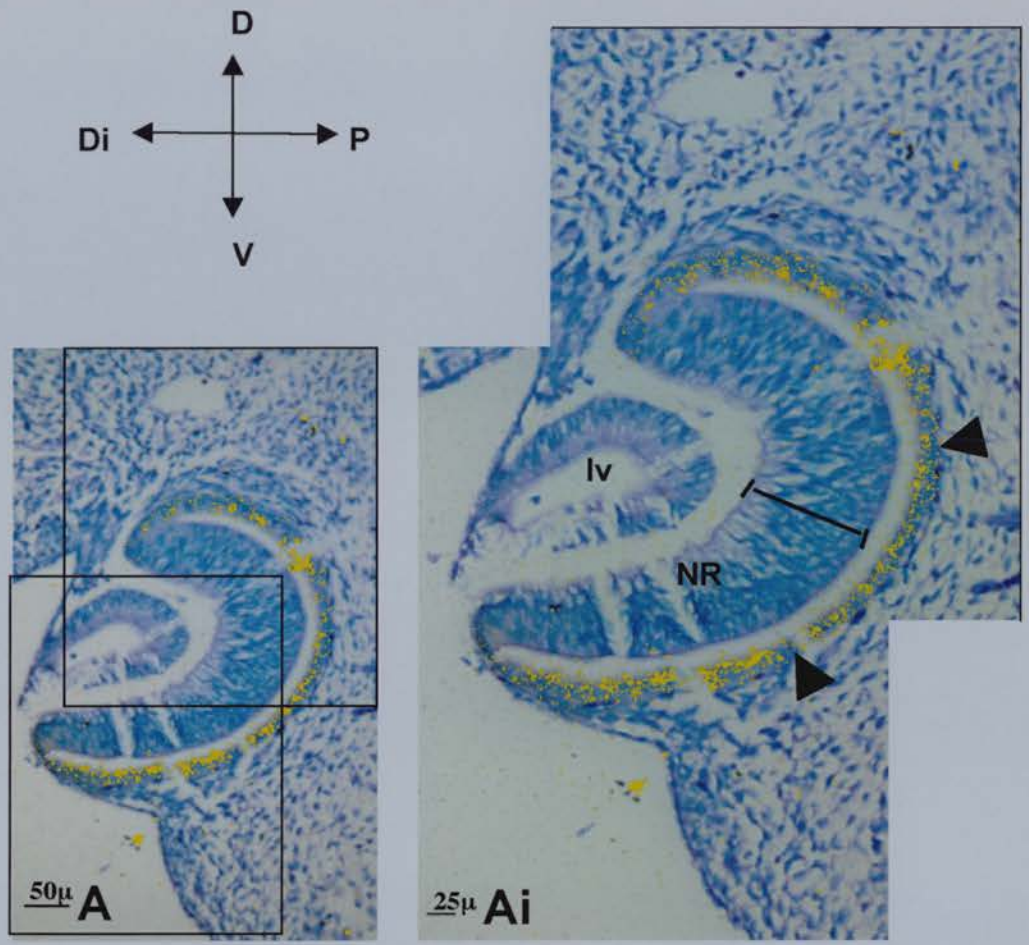


Fig 4.8 *Trp2* expression in eye region of E10.5 wildtype mouse A) Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). Ai) enlargement of the retina. NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

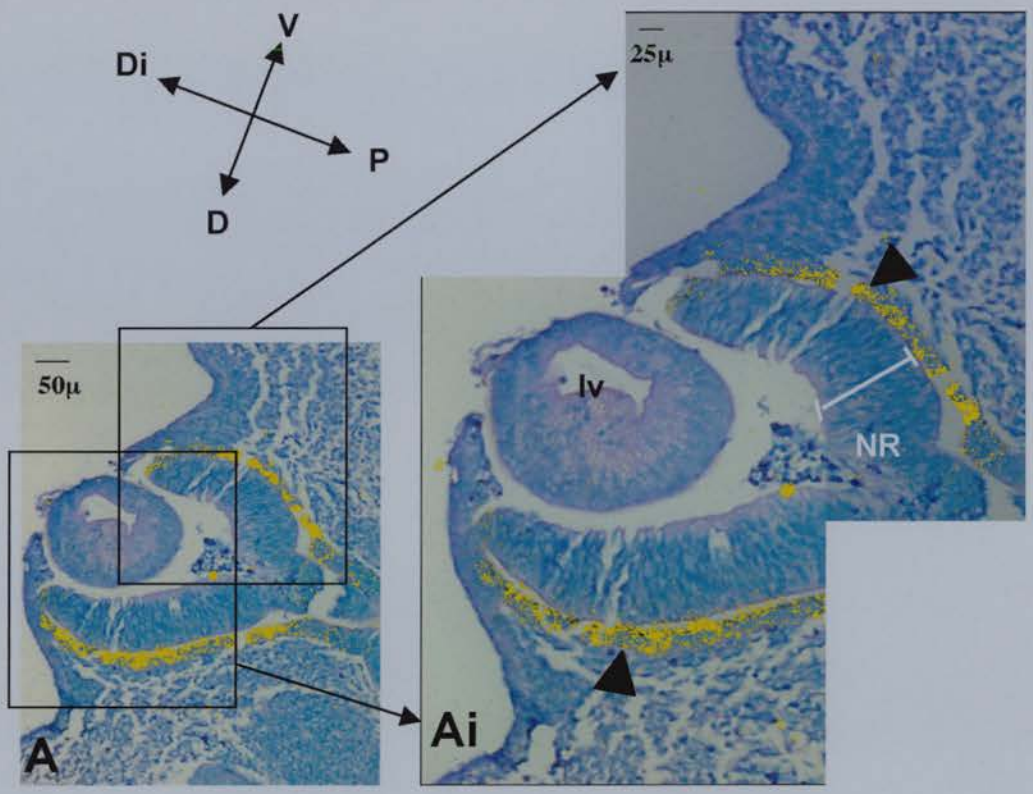


Fig 4.9 *Trp2* expression in eye region of E11.5 mouse from the transgenic line A81 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. Ai) enlargement of the retina. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

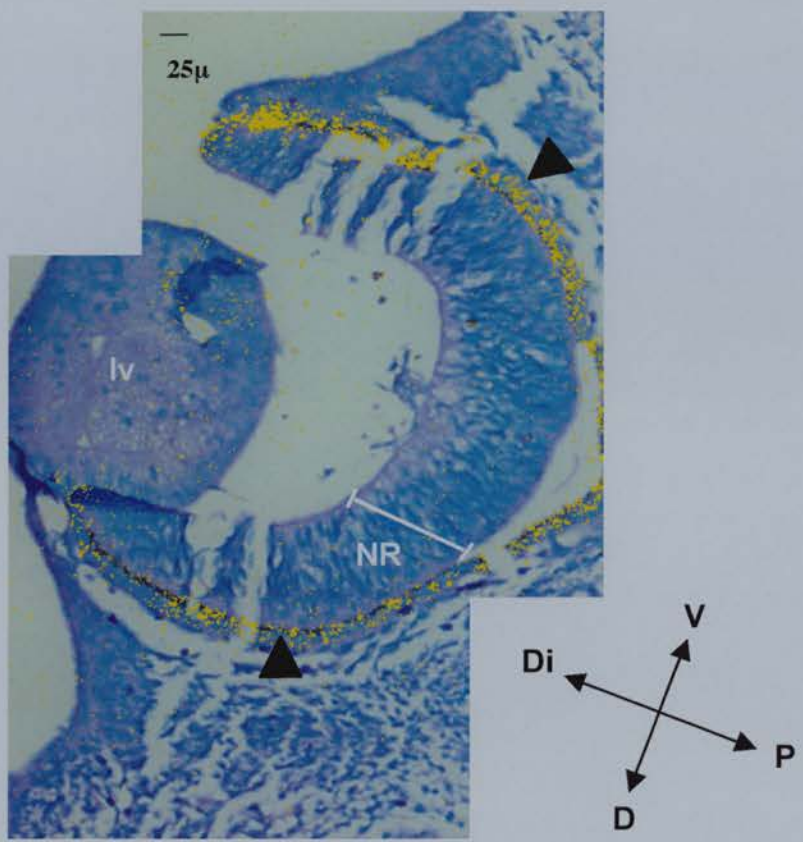


Fig 4.10 *Trp2* expression in eye region of a wildtype E11.5 mouse. Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

4.2.4 Conclusions

Small patches of βGal activity were observed in the PRE of the p*Trp2*-driven line A81. No ectopic *Msx2* could be detected in the PRE at E10.5 or E11.5 by *in situ* hybridization and *Trp2* expression in the PRE appeared normal at these stages. The level of βGal activity required to produce a detectable signal may be considerably lower than the level of transcript detectable by S^{35} radioactive in situ. This difference in sensitivity between the two detection methods may explain why the patches of βGal were observed in the PRE but no ectopic *Msx2* could be detected. Sufficient βGal protein may have been synthesized from the transgene in some PRE cells to be detected but the level of *Msx2* transcript was beyond the level detectable by S^{35} radioactive in situ. To investigate the sensitivity of in situ hybridization compared to βGal activity expression of lacZ and *Msx2* transcript and βGal activity could be investigated in consecutive A81 eye sections.

Alternatively, the lack of detectable ectopic *Msx2* may be a result of transgene silencing by several potential mechanisms. The transgene may have become silenced following expression with only a small number of *Msx*2IRESβGeo transcripts being produced and their unusual nature may have resulted in rapid degradation. However, sufficient highly stable βGal protein may have been synthesized from the transgene transcripts in a few PRE cells to produce a detectable signal.

Firstly, transgenes can be silenced by so-called 'position effects' if the transgene inserts in or near transcriptionally inactive regions of chromatin (Martin and Whitelaw, 1996). However, the lack of transgene activity in 7 out of 8 *Trp1*-or *Trp2*-driven lines suggests that there may be another explanation. Secondly, silencing can be promoted by the insertion of tandem multiple-copy arrays of the transgene at single sites (Henikoff, 1998). Repeat-induced gene silencing is frequently manifested as a decrease in the proportion of cells that express the transgene, resulting in a variegated pattern of expression. Silencing of multiple copies of the p*Trp2Msx2*Iβgeo transgene in the PRE of A81 transgenic mice could explain the

patches of βGal expression observed. Multiple integrated copies may have silenced the transgene in the *Trp2*-driven lines A80.1, A81.3 and A88, which did not show any detectable βGal expression. The number of copies of the transgene integrated into each transgenic line could be estimated by probing for *Msx2* on a Southern blot and using another gene, for example *Trp2* as a reference for two copies.

Thirdly, transgenes comprised of mammalian cDNAs and prokaryotic reporter genes are particularly prone to repressive effects and it has been suggested that such sequences may serve as active foci for gene silencing (Clark *et al.*, 1997). Both *Trp1* and *Trp2* transgenes contained *Msx2* cDNA followed by the prokaryotic βGeo reporter gene. Silencing due to the viral IRES and prokaryotic βgeo sequences may explain why so many *Trp*-driven *Msx2* transgenic lines showed no transgene activity.

Fourthly, silencing of transgenes may occur during development or during transmission through the mouse germline. During development methylation of CpG islands in the promoters of some genes suppresses their expression (Jones, 1999). Analyzing the CpG content and methylation state of the *Trp2* promoter in the integrated transgene may reveal whether methylation is inducing its silencing. This could be established by restriction digests on transgenic genomic DNA and plasmid DNA using methylation-sensitive isoschizomers. Developmentally associated gene regulatory mechanisms must be reset during sexual reproduction, but it is now becoming clear that some regulatory states can be inherited meiotically. Meiotic inheritance of epigenetic states has been observed in mammals (Morgan *et al.*, 1999) and in *Drosophila* and fission yeast which do not methylate their DNA (Cavalli and Paro, 1998; Grewal and Klar, 1996).

In a few cells in the PRE the transgene may have escaped silencing and clonal expansion of these cells produced the βGal-positive patches in the PRE. The βGal positive PRE cells do not appear to be any different from βGal -negative PRE cells. However, it would be interesting to investigate whether the βGal-positive PRE cells express *Msx2*, *Trp2* and Mitf. This could be examined by serial sections stained for

βGal and *in situ* hybridization for *Msx2,Trp2* and *Mitf* or immunohistochemistry for βGal, Msx and Mitf.

Finally, the transgene may have been active in the PRE of several of the *Trp1*- and *Trp2*-driven lines, but these did not show any βGal activity because of poor translation from the IRES. This could be investigated by *in situ* hybridization or immunohistochemistry for ectopic *Msx2* in the PRE of the mice from lines A80.1, A81.3 and A88.

To attempt to improve *Msx2* transgene expression the IRES/βgeo sequences were removed from the transgene and transgenic mice generated with the new construct.

4.3 Producing pTrp2Msx2 transgenic mice without the IRES/Bgeo cassette

4.3.1 Cloning steps

To produce transgenic mice expressing *Msx2* under control of the *Trp2* promoter the *Trp2*-promoter-intron-*Msx2* fragment was recloned from p*Trp2Msx2*IRESβgeoSV40 into pCI. p*Trp2Msx2*IRESβgeoSV40 was digested with *XbaI* and *XhoI* to yield the predicted 4923bp *Trp2*-promoter-intron-*Msx2* fragment and 5777bp *XbaI* IRESβgeo fragment, although these could not be resolved on a 1% TAE agarose gel. pCI was digested with *XbaI* and *XhoI*. Following separation on a 1% TAE agarose gel the pCI and *pTrp2Msx2/* IRESβgeo fragments were extracted using a Gel Extraction Kit (Quiex) (Fig 4.11).

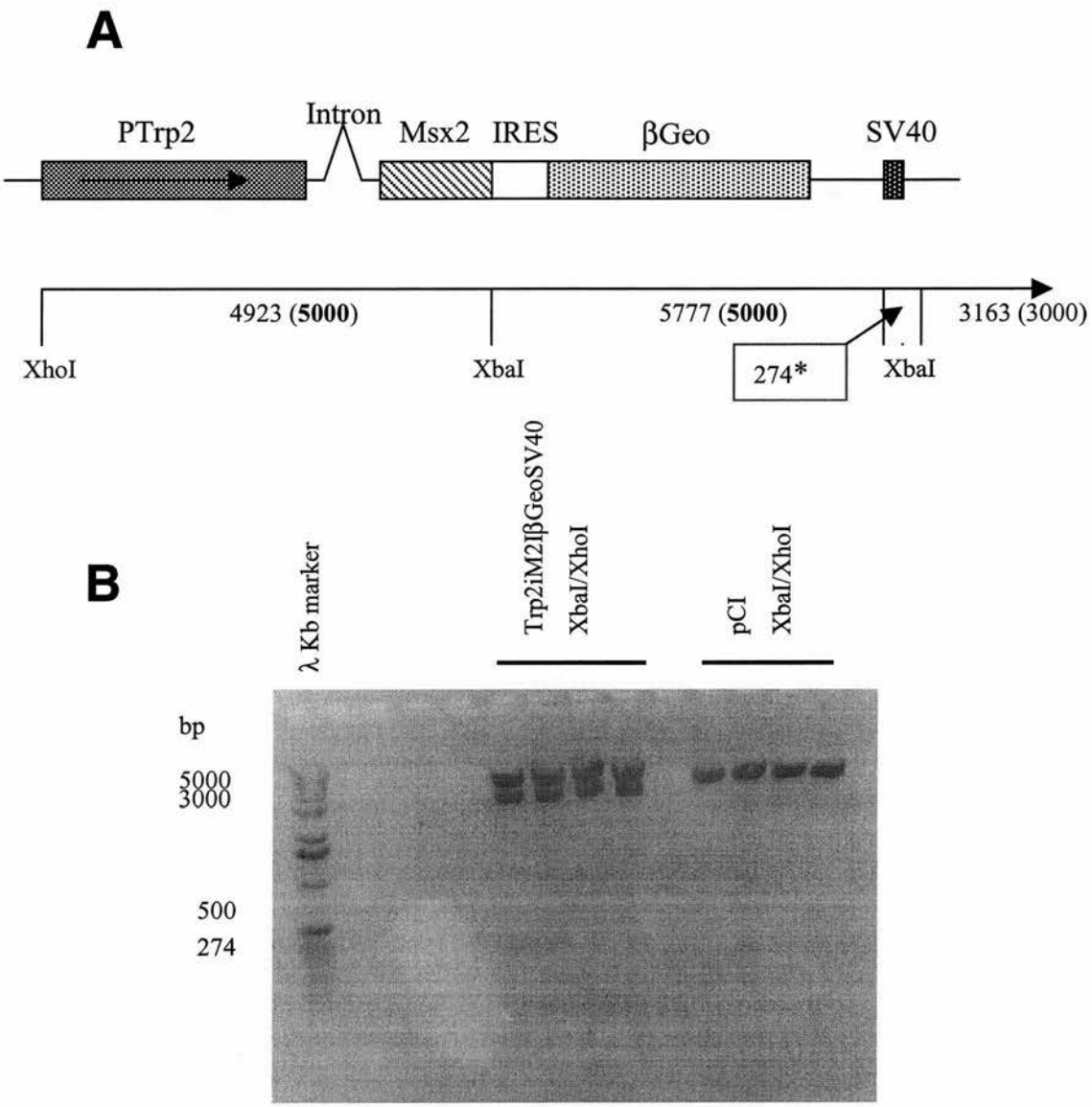


Fig 4.11 . A) Graphical representation of *pTrp2iM2*β*GeoSV40* construct showing restriction enzyme sites and predicted sizes of fragments following digestion, in bp. Sizes in brackets are the approximate size of the fragments actually observed. Sizes in bold correspond to broad bands which may contain two similarly sized fragments. B) Restriction digest of *pTrp2iM2*β*GeoSV40* construct and pCI plasmid with *XbaI /XhoI*.

The *pTrp2Msx2/* IRESβgeo fragments were ligated to the pCI digested with *XbaI/XhoI* using Rapid DNA Ligation Kit (Boehringer Mannheim). Only the 4923bp *XbaI/XhoI Trp2*-promoter-intron-*Msx2* fragment will insert into the *XbaI/XhoI* digested pCI. The ligation mixture was transformed into E.Coli by electroporation. The cloning was verified by digestion with *XbaI* and *XhoI* which released the 4923bp *pTrp2Msx2* fragment and *NsiI* which releases a 3278bp fragment including sites in *Msx2* and *pTrp2*. Analysis of a complete restriction digest confirmed that this cloning step had been successful (Fig 4.12)

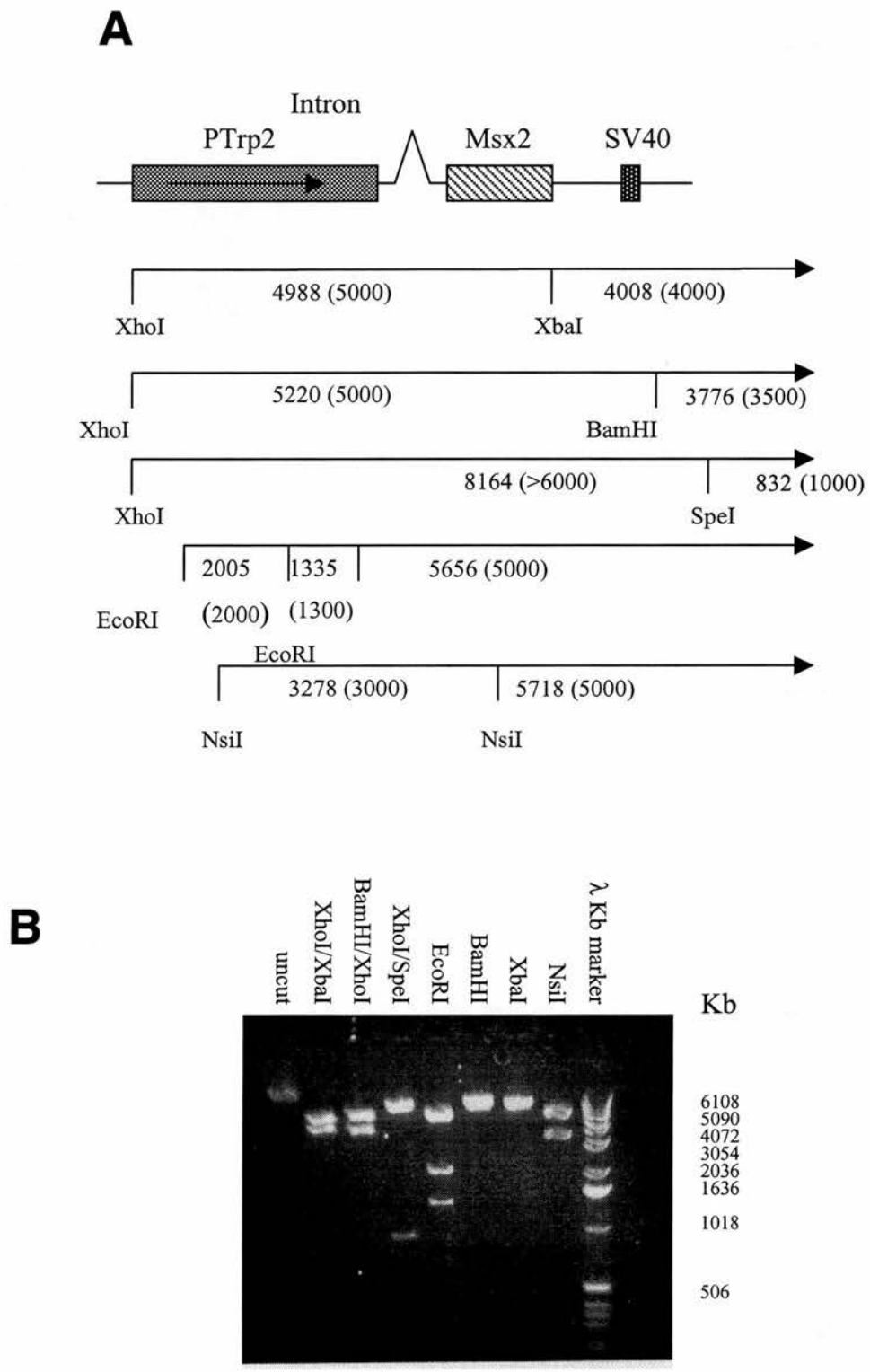


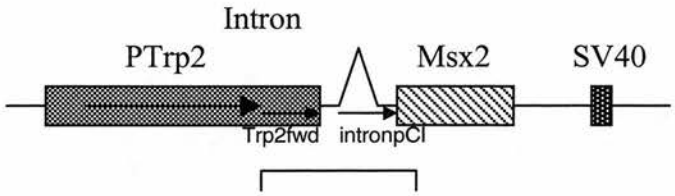
Fig 4.12 A) Graphical representation of the p*Trp2Msx2* construct with restriction sites. B) restriction digest of the p*Trp2Msx2* construct.

A total of 10μg of p*Trp2Msx2*IRESBGeoSV40 DNA was purified and digested with *XhoI* and *BamHI* to release the 5220bp transgene of p*Trp2Msx2*SV40. The p*Trp2Msx2* transgene was extracted from the gel using a Quiex Gel Extraction Kit and phenol/choloroform extraction, following purified the transgene was microinjected by L.Marshall into the pronucleus of CBA x C57BL/6 fertilized eggs which were then re-implanted into 10 pseudopregnant CD1 females.

4.3.2 Sequence analysis of m*Msx2* expression construct (p*Trp2Msx2*)

To design unique primers for tail tip PCR analysis of mice carrying the modified p*Trp2Msx2* transgene the section between the *Trp2* promoter and the start of the *Msx2* gene was manually sequenced using P^{33}. The first sequencing reaction used a primer to the 3' end of the *Trp2* promoter (Fig 4.13). The sequence obtained from this reaction, through the intron into the pCI cloning vector was used to design another primer to the intron-pCI breakpoint for a further sequencing reaction. From this second reaction sequence through the multiple cloning site of the pCI into the 5' end of the *Msx2* gene was obtained (Fig 4.13).

A



B

Trp2

agacagaaaccaggtgggacgccgggccagggctcgacggtatcgataagcttgatatcgaattcctgc

intron

agaagttggtcgtgaggcactgggcaggtaagtatcaaggttacaagacaggtttaaggagaccaatag

aaactgggcttgtcgagacagagaagactcttgcgtttctgataggcacctattggtcttactgacatccactt

gccttctctccacaggtgtccac*****gttcaattacattaaggctagagtacttaatacgactcactataggc

Msx2

tagcctcgacggtatcgataagcttcctctttaaacaatcggctttaattacgccttagacttttcagtttgggcg

attataacccttgagggatcgcctaataacaactctgctgactgctcctgtaattaactcctaatttatttcaaac

ggggcggggaagggccccaagcctctccaggggagagccaatcgggtgggcagcgtccgtggcgtca

ggagcagggccgtcgcca

Fig 4.13. A) Graphical representation of p*Trp2iMsx2* construct showing positions of primers used for sequencing and the section of the construct sequenced. B) Sequence obtained from the 3' end of the *Trp2* promoter through the intron into the 5' end of the *Msx2* coding region. * represents bases which could not be resolved on the sequencing gel.

4.3.3 p*Trp2Msx2* transgenic lines

In total 35 mice were born from 4 embryo transfers. Tail tip PCR analysis showed that two males and one female of these mice were carrying the transgene. The two male founders were designated A204, A205 and the female A203.
The founders A204 and A205 were crossed with CBA x C57BL/6 F1 mice. Litters from each line were allowed to go to term and the sperm from transgenic males was frozen for further study (C586.1 from A204, C587 from A205, C584 and C584.3 from A203). Embryos from the lines A204 and A205 were collected, fixed and embedded for *in situ* analysis (Table 4.1). Embryonic tail tip PCR revealed that 10 out of the 33 embryos were carrying the transgene.

Line	age	No. of embryos	No. of tg embryos
A205	E10.5	18	6
A205	E11.5	10	2
A204	E10.5	5	2

Table 4.1 Ages and transgenic status of embryos collected from the lines A204 and A205.

4.3.4 Morphological analysis of eyes of the transgenic lines A204 and A205

In E10.5 transgenic embryos from the lines A204 and A205 no morphological differences are observed in the overall size and shape of the optic cup and lens, or the thickness of neural retina and pigmented retinal epithelium compared with wild type (See Figs, 4.14, 4.15, 4.16, 4.17, 4.18, 4.19, 4.20, 4.21). Since the transgene no longer contains the βGal reporter gene one of the few ways available to investigate transgene activity is by *in situ* hybridization for ectopic expression of *Msx2* in the PRE

4.3.5 *In situ* analysis of *Msx2* in embryos from transgenic lines A204 and A205

Sections of wax-embedded embryos of wildtype and transgenic A204 and A205 mice (E10.5d) were hybridized with a S^{35} radiolabelled *Msx2* RNA probe in two independent *in situ* hybridizations. Native *Msx2* mRNA was detected in the lens vesicle of E10.5d wildtype and transgenic embryos from lines A204 and A205, (see Figs 4.14, 4.15, 4.16). No ectopic *Msx2* transcripts could be detected in the PRE of the embryos in lines A204 and A205 carrying the transgene, (see Figs 4.14 and Fig 4.15). The level of ectopic *Msx2* expression from the transgene in the PRE may have been too low to be detectable by *in situ* hybridization.

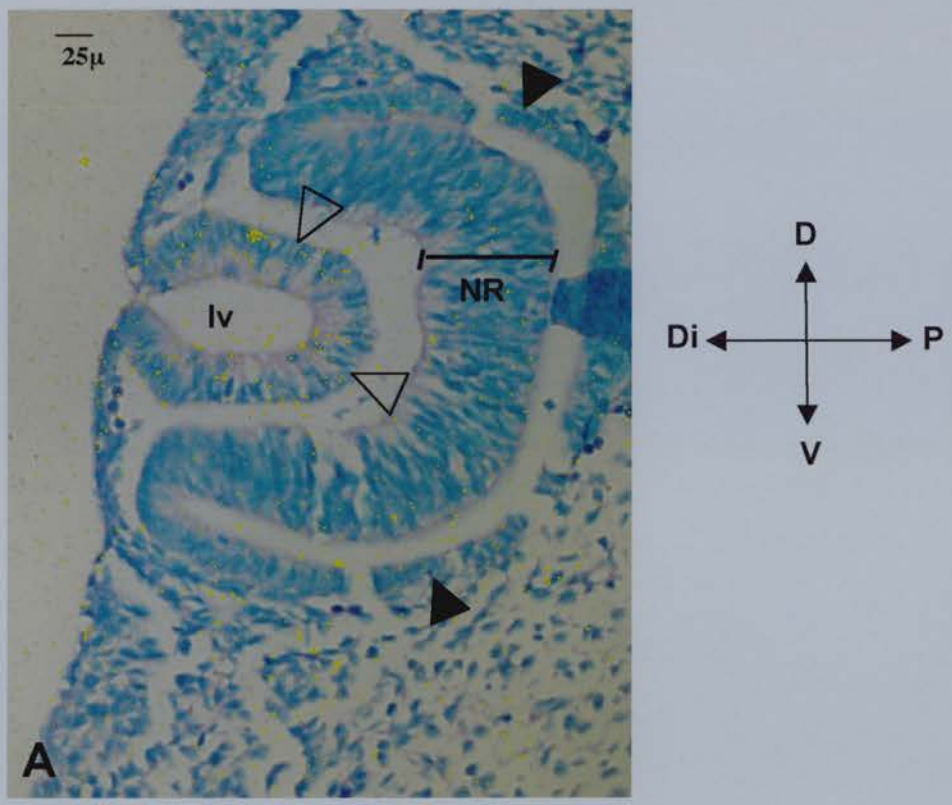


Fig 4.14 *Msx2* expression in eye region of E10.5 mouse from the transgenic line A204 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the lens vesicle (open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

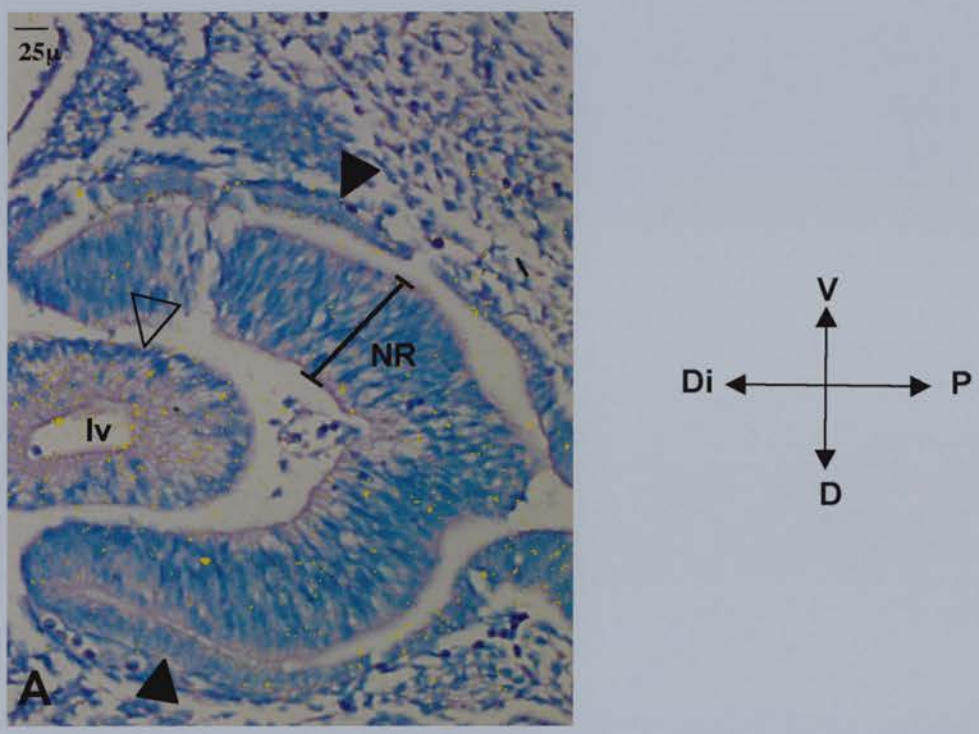


Fig 4.15 *Msx2* expression in eye region of E10.5 mouse from the transgenic line A205 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the lens vesicle(open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal

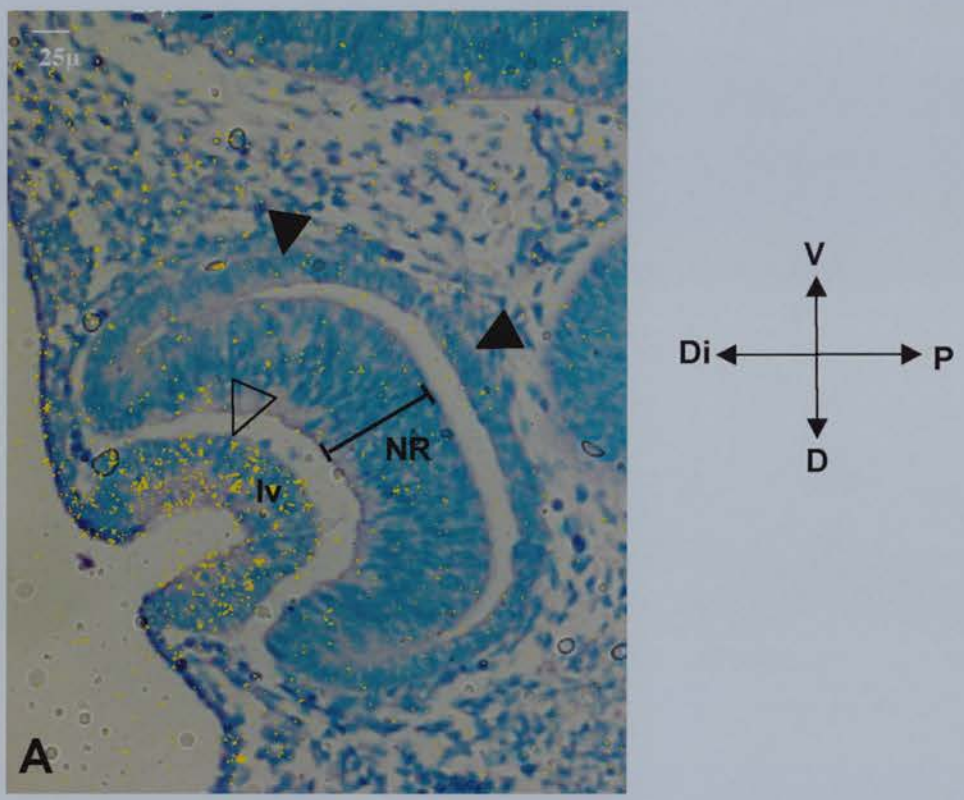


Fig 4.16 *Msx2* expression in eye region of E10.5 wildtype mouse. A) Transverse section through the posterior half of the eye. *Msx2* expression was observed in the lens vesicle, (open arrow). Ai) enlargement of the retina. No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal

4.3.6 *Trp2* expression is normal in embryos from transgenic lines A204 and A205

Alternatively the transgene may have been subject to repression, for example autorepression. To investigate this *Trp2* expression was analyzed in transgenic embryos from lines A204 and A205. Sections of wax-embedded embryos of wildtype and transgenic A204 and A205 mice (E10.5d) were hybridized with a S^{35} radiolabelled *Trp2* RNA probe in two independent *in situ* hybridizations. Expression of *Trp2* in the PRE of E10.5d embryos in lines A204 and A205 carrying the transgene appeared normal in comparison with expression of *Trp2* in their wild type littermates (Figs 4.17, 4.18 and 4.19). The normal level of *Trp2* expression in transgenic embryos from the lines A204 and A205 suggests that the lack of *Msx2* transgene activity was not a result of transgene silencing by autorepression.

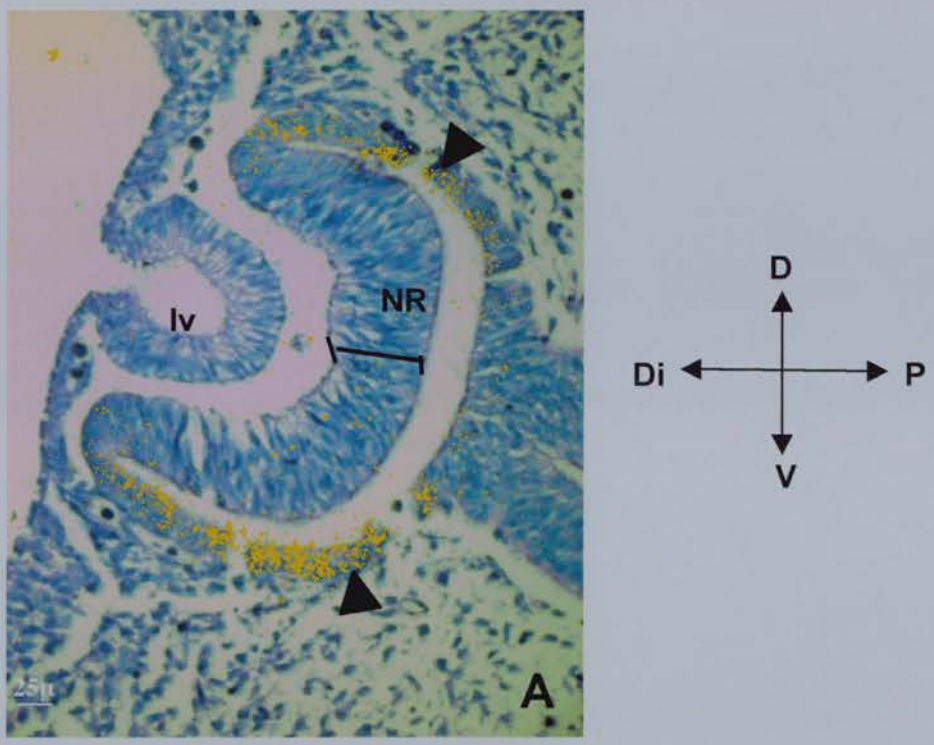


Fig 4.17 *Trp2* expression in eye region of E10.5 mouse from the transgenic line A204 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

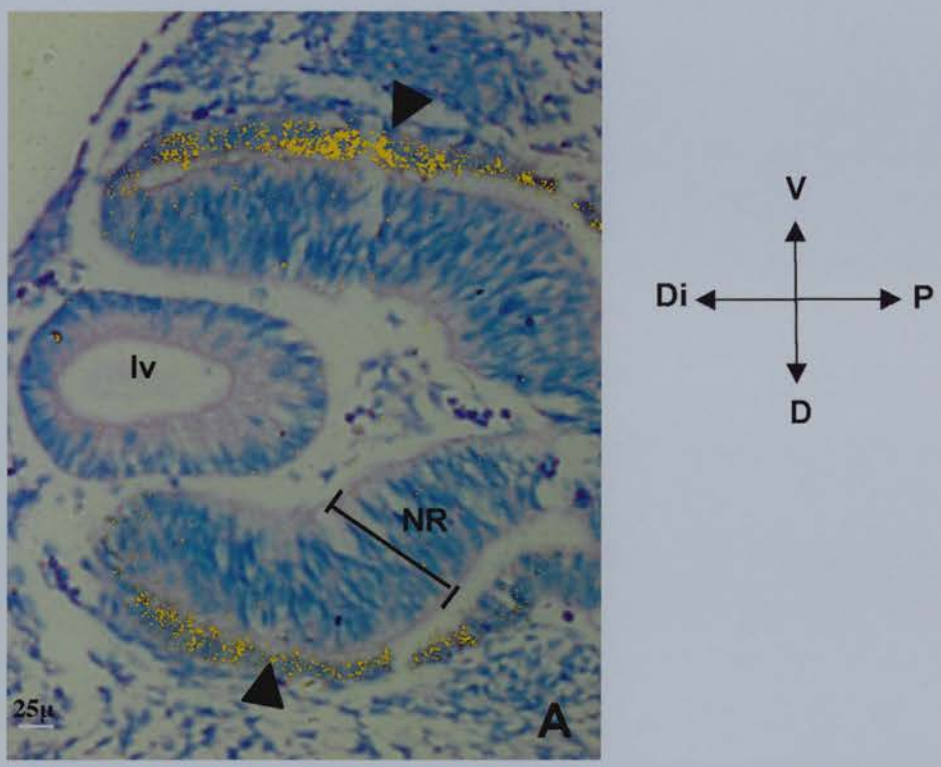


Fig 4.18 *Trp2* expression in eye region of E10.5 mouse from the transgenic line A205 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

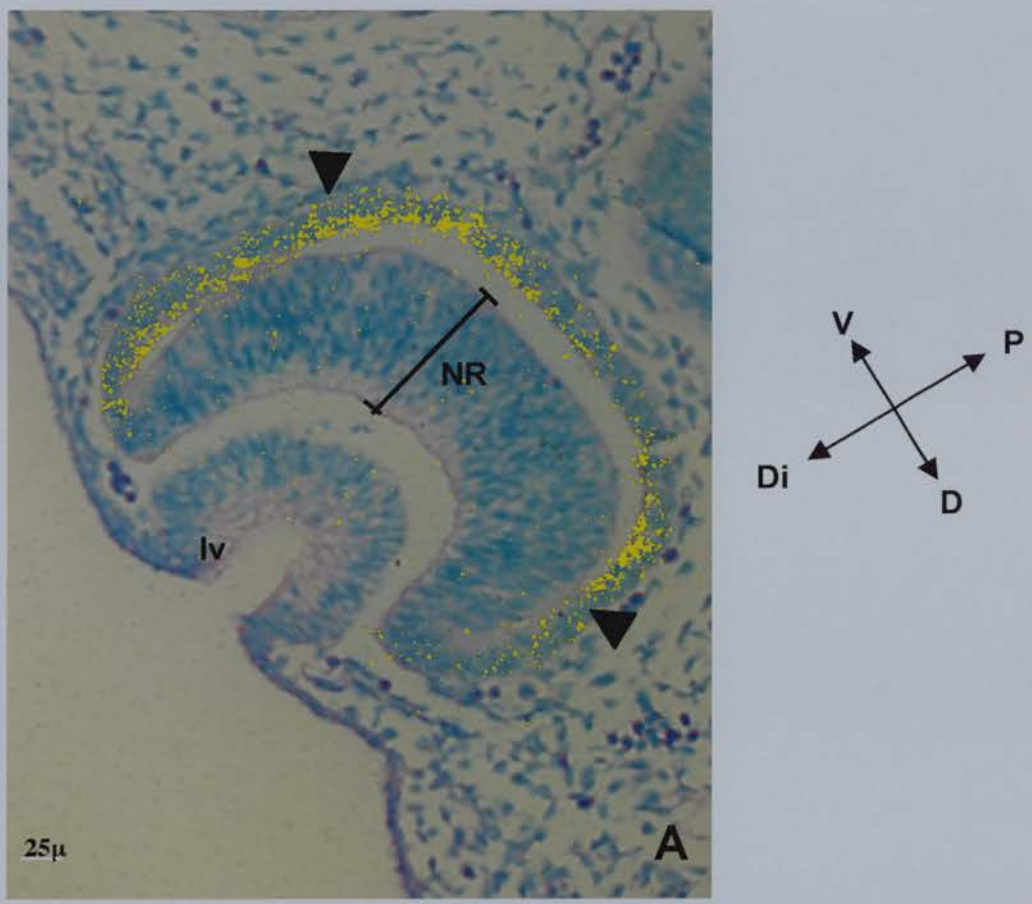


Fig 4.19 *Trp2* expression in eye region of wildtype E10.5 mouse A) Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle.

In the *in vitro* assay a cellular effect observed when *mMsx2* is ectopically expressed in chick PRE cells is a downregulation of Mitf protein in m*Msx2*-transfected cells. To investigate whether, despite being undetectable by *in situ* hybridization, the *Msx2* transgene is active and has a similar biological effect in the PRE of A204 and A205 transgenic mice *Mitf* expression was analyzed.

4.3.7 *Mitf* is normal in embryos from transgenic line A204

Sections of wax-embedded embryos of wildtype and transgenic A204 mice (E10.5d) were hybridized with a S^{35} radiolabelled *Mitf* RNA probe in two independent *in situ* hybridizations. Native expression of *Mitf* in the PRE of E10.5d embryos of the line A204 carrying the transgene was indistinguishable from *Mitf* expression in their wildtype littermates, (see Figs 4.20 and Fig 4.21). If the transgene is active in the line A204 then it does not appear to have any effect on *Mitf* expression.

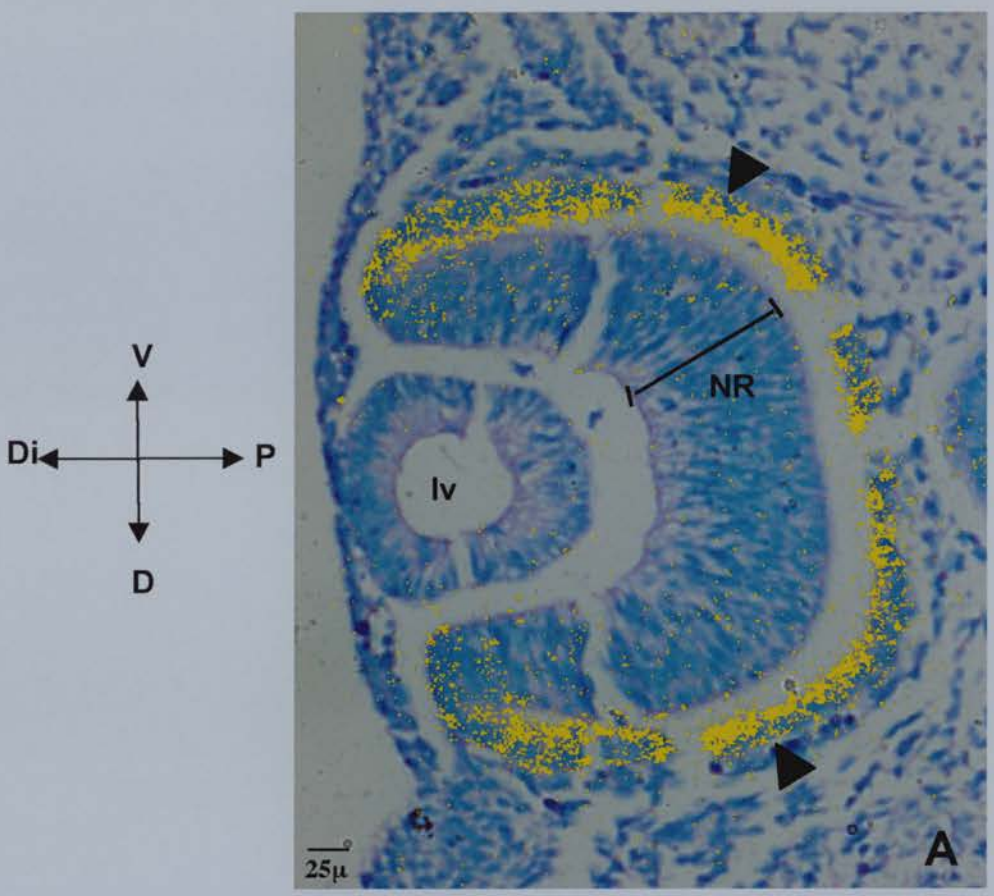


Fig 4.20 *Mitf* expression in eye region of E11.5 mouse from the transgenic line A204 which PCR analysis showed were carrying the transgene. A) Transverse section through the posterior half of the eye. *Mitf* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

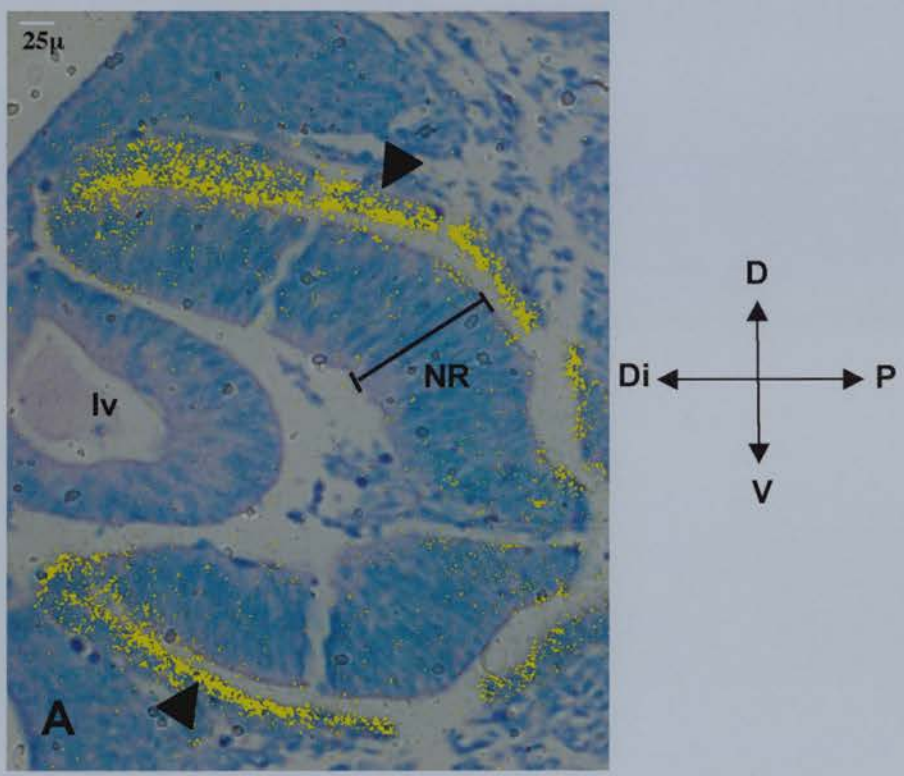


Fig 4.21 *Mitf* expression in eye region of E10.5 wildtype mouse. A) Transverse section through the posterior half of the eye. *Mitf* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle.

4.3.8 Conclusions

There is no evidence of *Msx2* transgene activity in the transgenic lines A204 or A205. The normal expression of *Trp2* in the eyes of transgenic embryos from both A204 or A205 suggests that transgene autorepression does not explain the lack of transgene activity.

The potential silencing influence of the IRES/βGeo sequences have been removed, but as in the lines analyzed previously, there are several other silencing mechanisms which may explain the lack of transgene activity. Transgene silencing may have resulted from either transgene insertion into a transcriptionally silent section of DNA or multiple integrated copies of the transgene or as a result of germline transmission. As described previously, transgene copy number could be estimated by a Southern blot. To examine both a greater number of different transgene integration events and whether the transgene was being silenced during germline transmission, transient transgenic embryos were generated using the p*Trp2Msx2* construct.

4.4 Producing pTrp2Msx2 transient transgenic embryos

The p*Trp2Msx2* construct was microinjected by L.Marshall into the pronucleus of CBA x C57BL/6 fertilised eggs which were then re-implanted into 6 pseudopregnant CD1 females; the embryos were collected 10.5 days after transfer; in two females no embryos had developed; two litters only contained two embryos each and the embryos in one of these litters showed delayed development. Two other litters contained six embryos each. A total of 16 embryos were collected fixed in 4% PFA and embedded in wax.

The tail tip PCR analysis of these embryos showed that the transgene had integrated into 3 embryos; 2.5, 3.5 and 4.4 (Fig 4.22) which were strongly positive to the transgene specific primers.

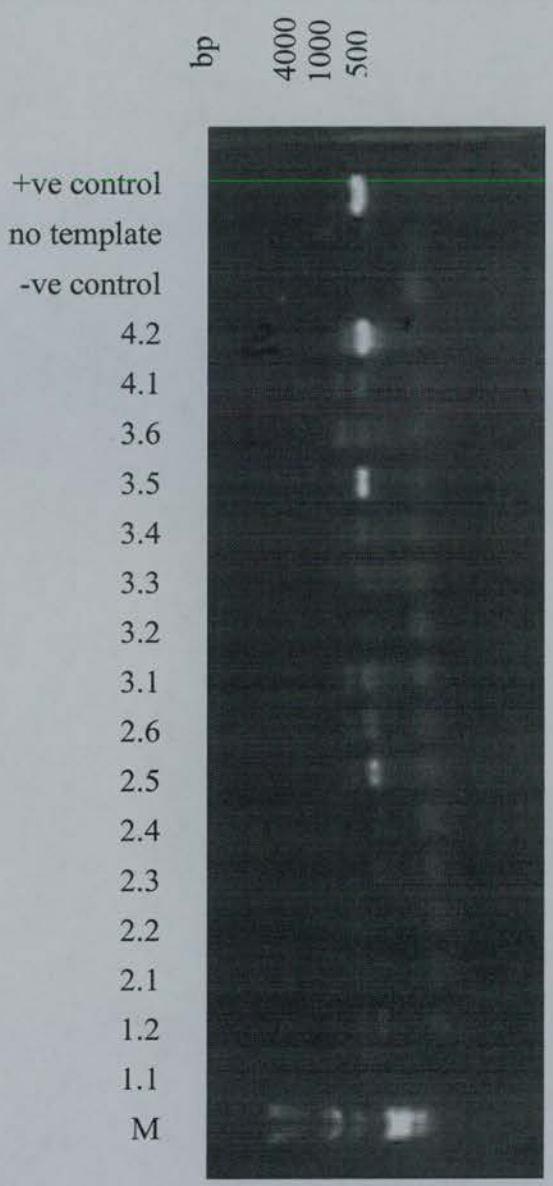


Fig 4.22. Identification of *Trp-2* driven *Msx2* transgenic mice. DNA extracted from tail biopsies of embryos collected from pseudopregnant CD1 mice implanted with CBA x C57BL/6 fertilised eggs injected with Trp2iM2SV40. The transgene was detected by PCR using primers Trp2forward and Msx2/3 which amplify a 500bp fragment. PCR products were observed by electrophoresis on a 1.2% agarose-gel containing EtBr.

4.4.1 Morphological analysis of eyes of the transient transgenic embryos

No gross morphological differences were observed between wild type embryos of a comparable developmental stage and transient transgenic embryos, 2.5, 3.5 and 4.4 in the overall size and shape of the optic cup and lens or the thickness of neural retina and pigmented retinal epithelium (Figs 4.24, 4.25, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32, 4.33 and 4.34). The thinner pigmented retinal epithelium in the sections of one of the eyes of transgenic embryo 2.5 and distorted optic cup in the sections of one of the eyes of transgenic embryo 3.5 (Figs 4.24, 4.27, 4.31 and 4.32) appear to be artifacts of the plane of section.

4.4.2 *In situ* analysis of *Msx2* in transient transgenic embryos

To investigate activity of the transgene in the transient transgenic embryos expression of *Msx2* was analyzed by *in situ* hybridization. Sections of wax-embedded embryos of wildtype and transgenic A204 and A205 mice (E10.5d) were hybridized with a S^{35} radiolabelled *Msx2* RNA probe. Native *Msx2* transcript could be detected in the branchial arches (data not shown), where it is normally expressed at a high level. In the PRE of the E10.5 transient transgenic embryos 2.5, 3.5 or 4.4 no ectopic *Msx2* was detected (Figs 4.23, 4.24, 4.25). However, if the expression level of ectopic *Msx2* from the transgene in the PRE was low it may have been beyond the level detectable by *in situ* hybridization. Unfortunately, the number of eye sections from positive transient transgenic embryos was severely limited and the *Msx2 in situ* hybridization could not be repeated.

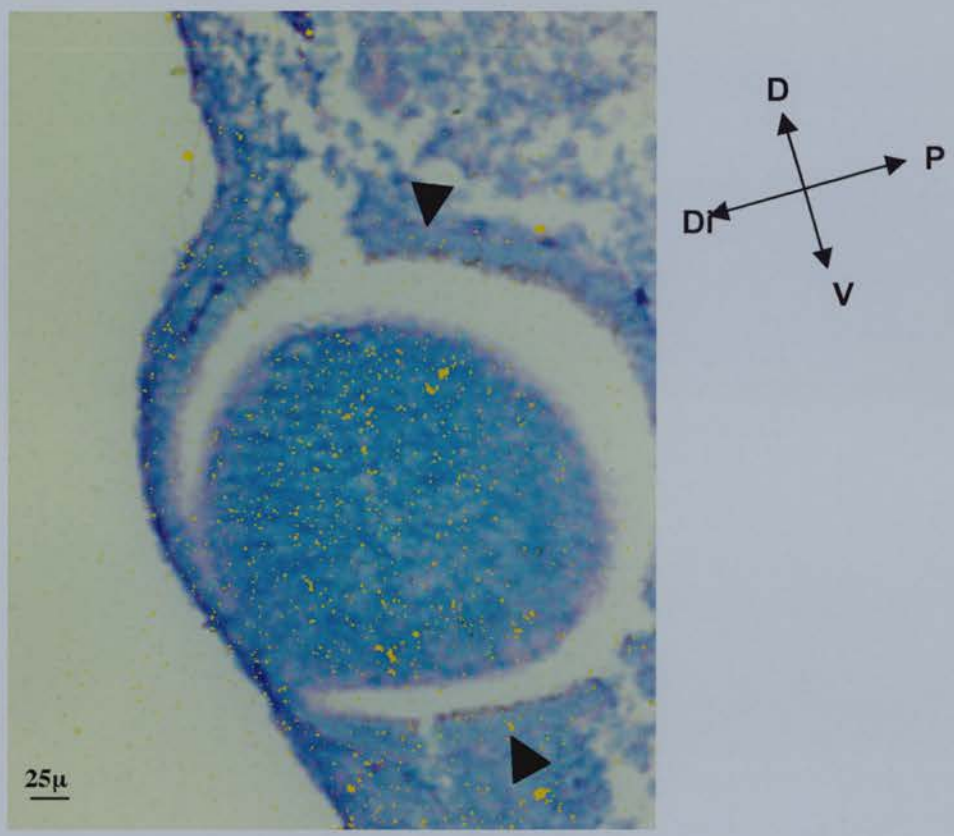


Fig 4.23 *Msx2* expression in eye region of the E10.5 transient transgenic mouse 2.5 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Msx2* expression was observed in the branchial arches ventral to the eye, (not shown). No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

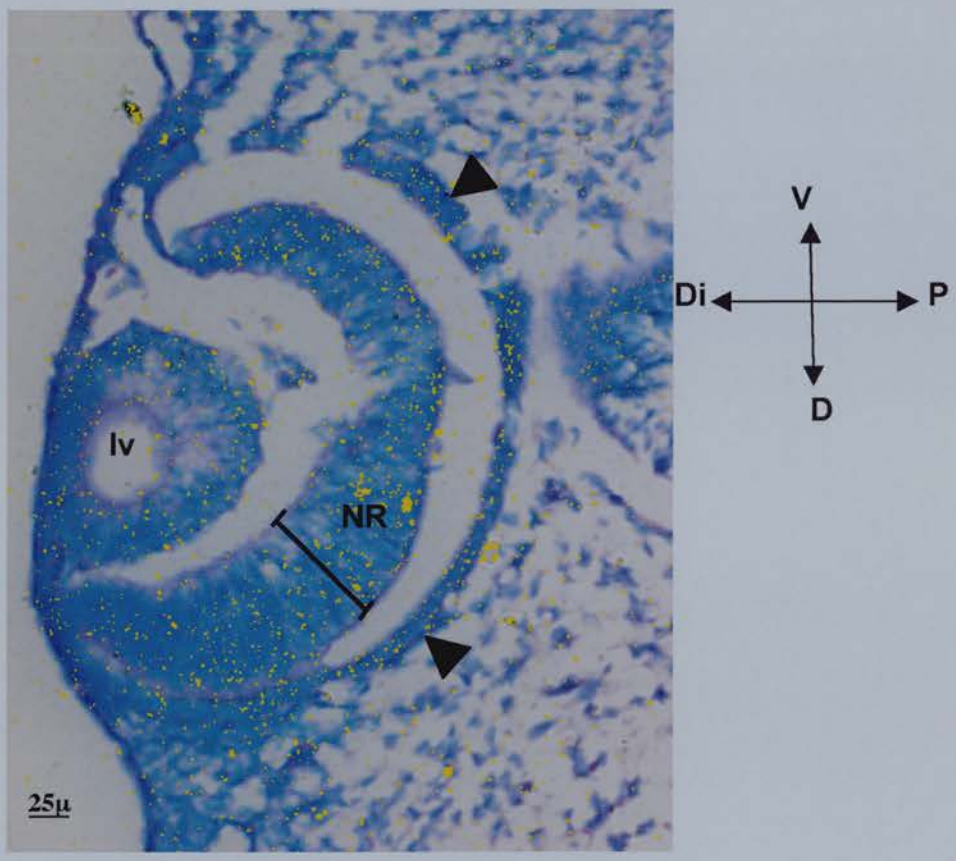


Fig 4.24 *Msx2* expression in eye region of the E10.5 transient transgenic mouse 3.5 which PCR analysis showed was carrying the transgene. Transverse section through the posterior half of the eye. *Msx2* expression was observed in the branchial arches buds to the eye, (not shown). No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. Orientation; V=Ventral, D=Dorsal, P=Proximal, Di =Distal.

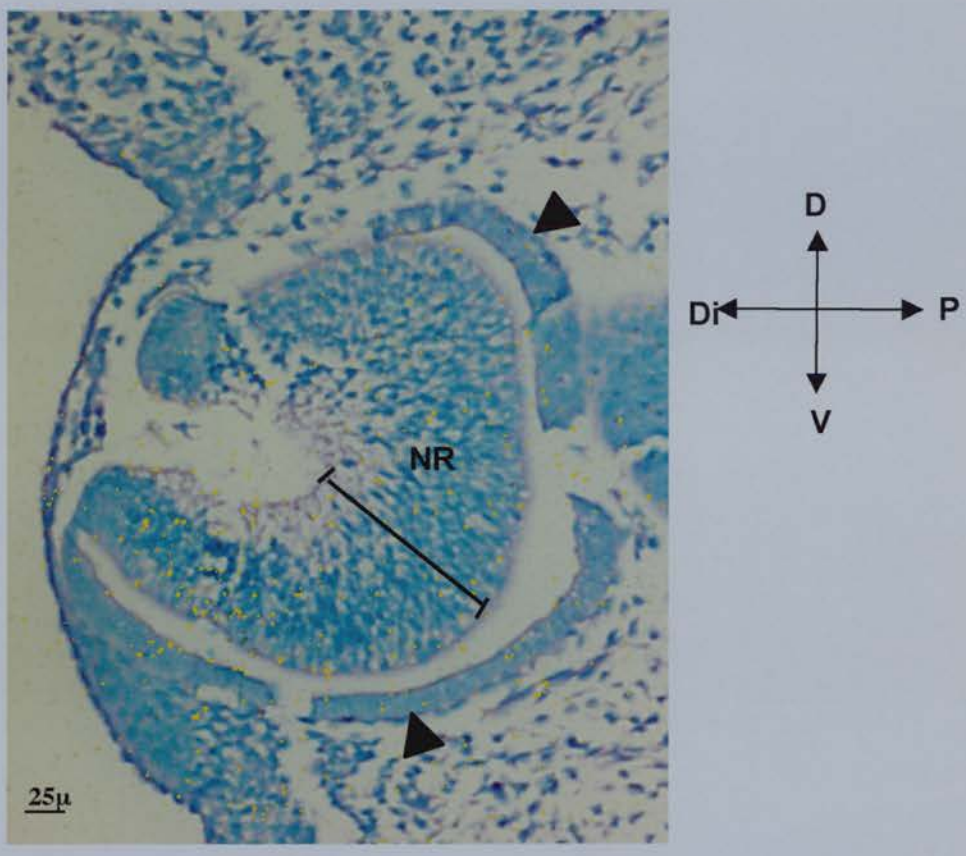


Fig 4.25 *Msx2* expression in eye region of the E10.5 transient transgenic mouse 4.2 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Msx2* expression was observed in the branchial arches ventral to the eye, (not shown). No *Msx2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

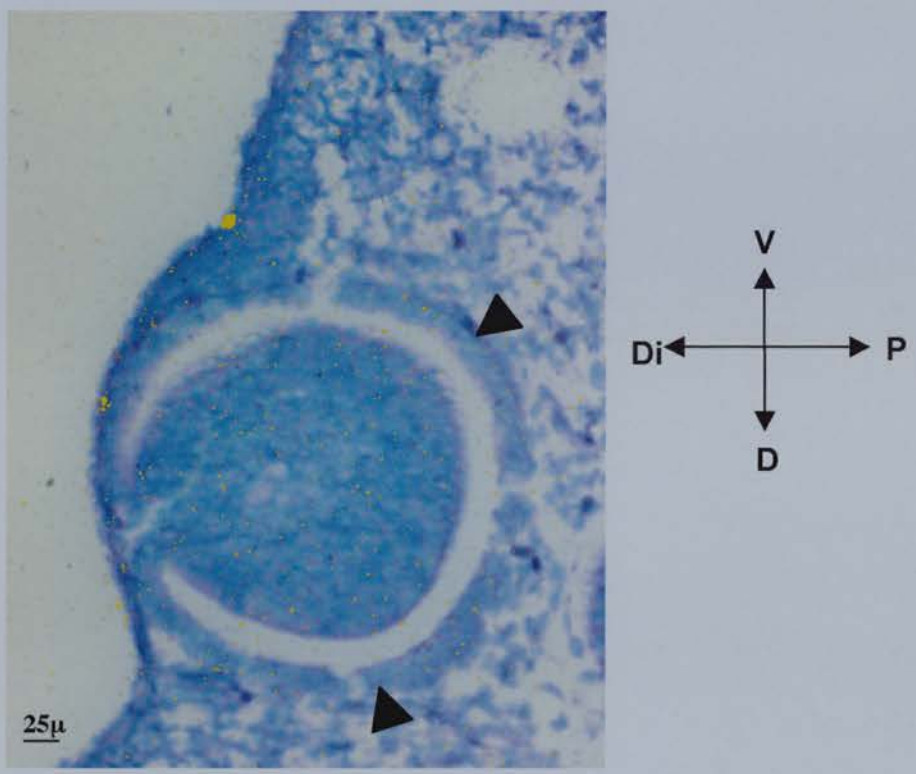


Fig 4.26 *Msx2* expression in eye region of a wildtype E10.5 littermate of the transient transgenic mice. Transverse section through the posterior half of the eye. *Msx2* expression was observed in the branchial arches ventral to the eye, (not shown). Pigmented retinal epithelium, (arrowheads). Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

4.4.3 *Trp2* expression is normal in transient transgenic embryos

To investigate autorepression by the transgene expression of *Trp2* was analyzed in the transient transgenic embryos. Sections of wax-embedded embryos of wildtype and transient transgenic embryos 2.5, 3.5 and 4.2 (E10.5d) were hybridized with a S^{35} radiolabelled *Trp2* RNA probe. Expression of *Trp2* in the transient transgenic mice, 2.5, 3.5 and 4.2, (see Figs 4.27, 4.28 and 4.29) was not significantly different to that in a wildtype embryo from the same litter (Fig 4.30). The lack of *Trp2* expression in the ventral PRE of the section of the transient embryo 3.5 (Fig 4.28) appears to be an artifact of the plane of section. The normal expression of *Trp2* in the transient transgenic embryos 2.5, 3.5 and 4.2 suggests that autorepression does not explain the lack of transgene activity.

4.4.3 *Mitf* expression is normal in transient transgenic embryos

Finally, as with the lines previously, *Mitf* expression in the transient embryos was investigated to check whether, despite the lack of detectable transgene activity a low level of ectopic *Msx2* expression was having a similar biological effect to that observed in the *in vitro* assay. Sections of wax-embedded embryos of wildtype and transient transgenic embryos 2.5, 3.5 and 4.2 (E10.5d) were hybridized with a S^{35} radiolabelled *Mitf* RNA probe. Expression of *Mitf* in the transient transgenic mice, 2.5, 3.5 and 4.2, (see Figs 4.31, 4.32 and 4.33) was not significantly different to that in a wildtype embryo from the same litter (Fig 4.34). If the transgene is promoting ectopic *Msx2* expression in the PRE of the transient transgenic embryos 2.5, 3.5 and 4.2 then it is not having an effect on *Mitf* expression. There is no evidence for *Msx2* transgene was activity in these transient transgenic mice.

A mutation may have been introduced into either the *Trp2* promoter or *Msx2* coding sequence during cloning. The integrity of the transgene could be checked by sequencing. To check the functional integrity of the transgene it could be transiently transfected into chick PRE cells and expression of *Msx2* transcript analyzed by RT-PCR or expression of the Msx2 protein examined by immunohistochemistry with an anti-Msx antibody.

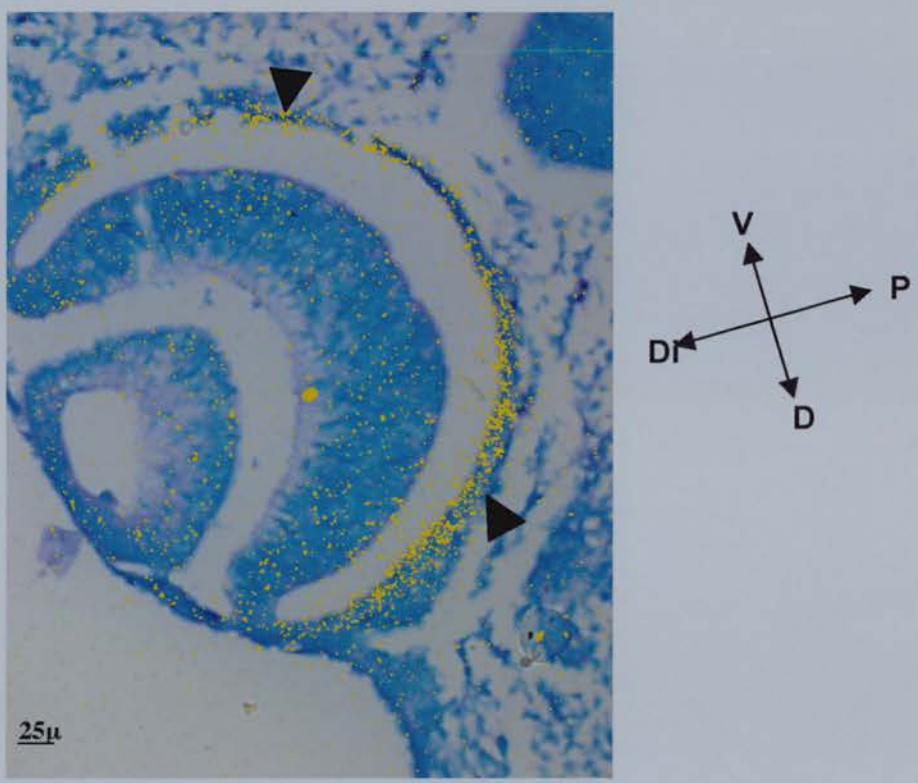


Fig 4.27 *Trp2* expression in eye region of the E10.5 transient transgenic mouse 2.5 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Trp2* expression is observed in the pigmented retinal epithelium, (arrowheads). V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

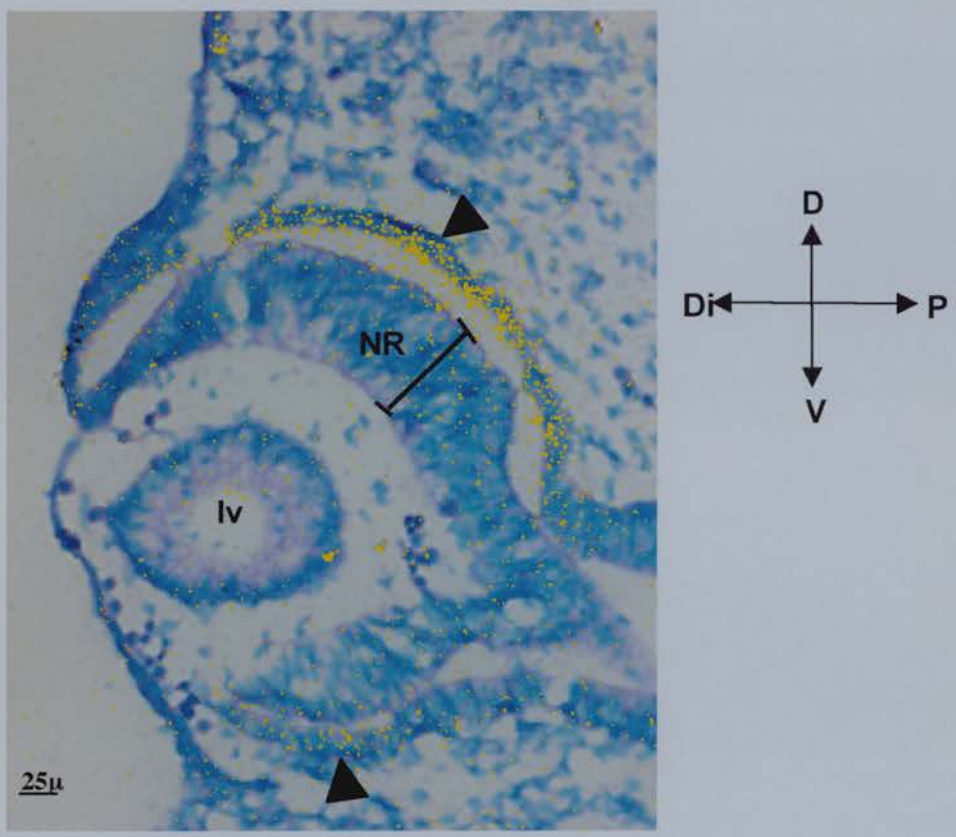


Fig 4.28 *Trp2* expression in eye region of the E10.5 transient transgenic mouse 3.5 which PCR analysis showed was carrying the transgene. Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

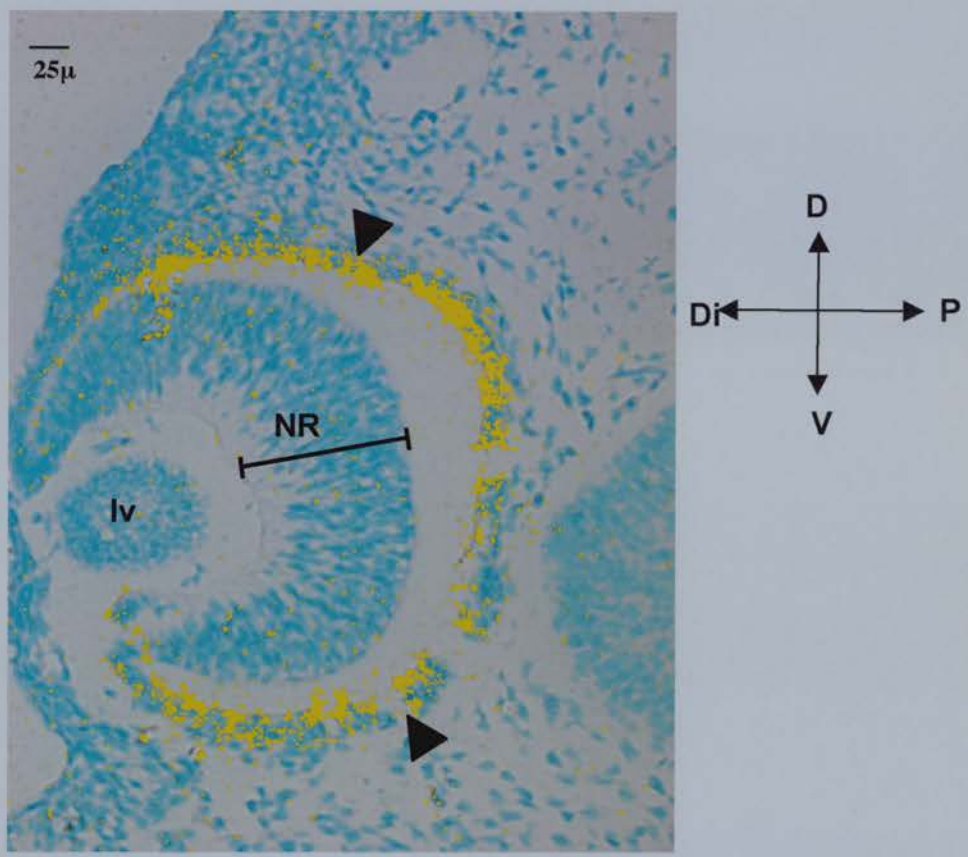


Fig 4.29 *Trp2* expression in eye region of the E10.5 transient transgenic mouse 4.2 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

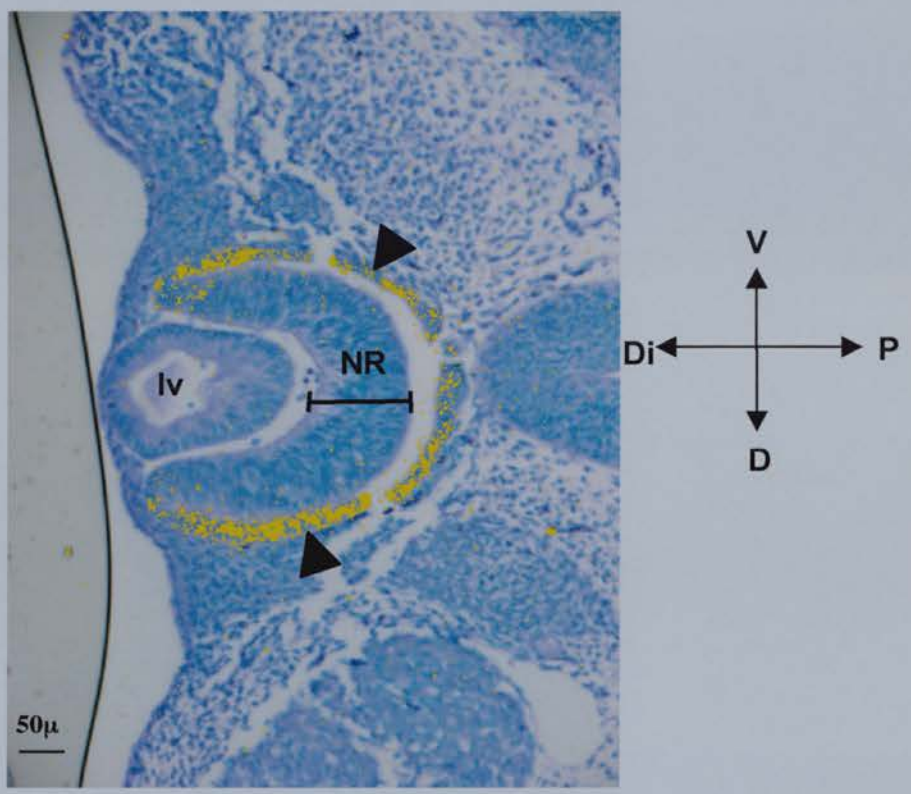


Fig 4.30 *Trp2* expression in eye region of a wildtype E10.5 littermate of the transient transgenic mice. Transverse section through the posterior half of the eye. *Trp2* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

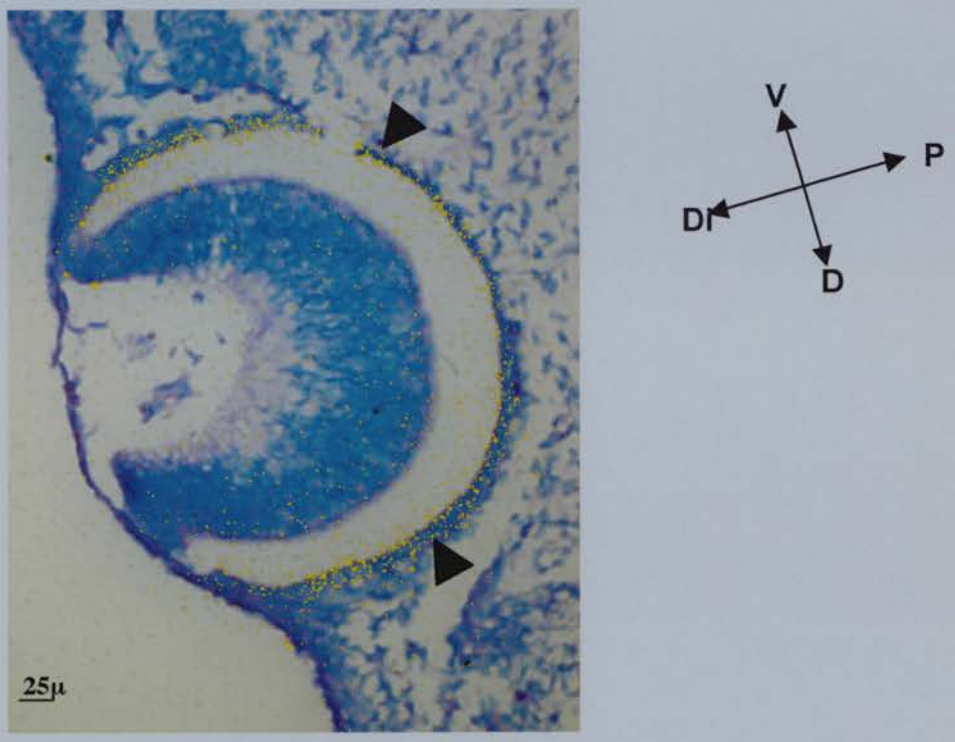


Fig 4.31 *Mitf* expression in eye region of the E10.5 transient transgenic mouse 2.5 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Mitf* expression was observed in the pigmented retinal epithelium, (arrowheads). Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

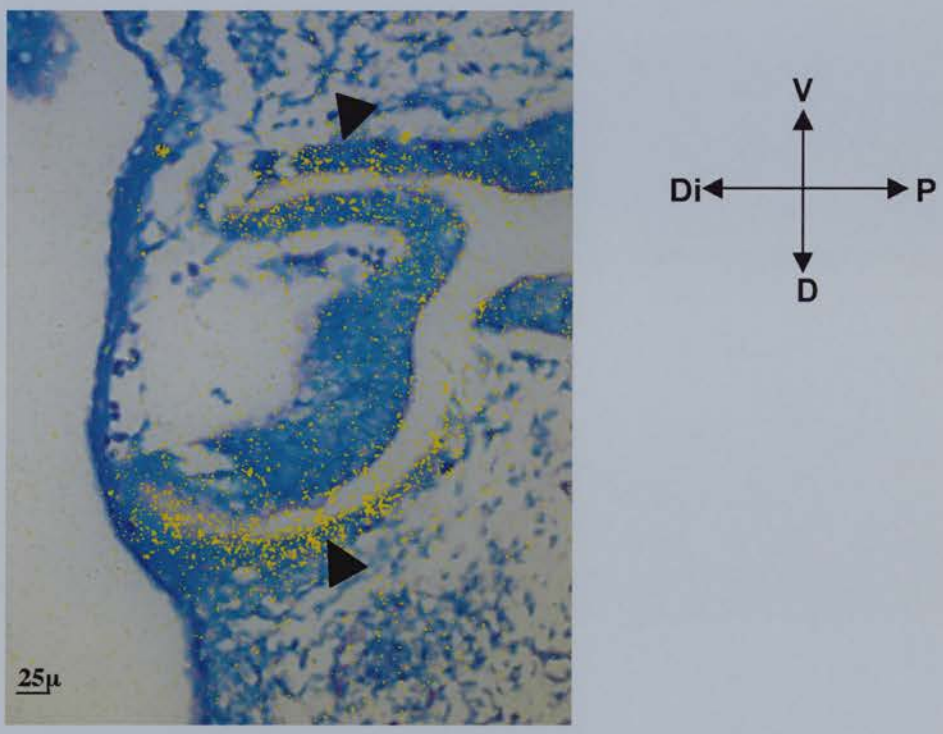


Fig 4.32 *Mitf* expression in eye region of the E10.5 transient transgenic mouse 3.5 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Mitf* expression was observed in the pigmented retinal epithelium, (arrowheads). Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

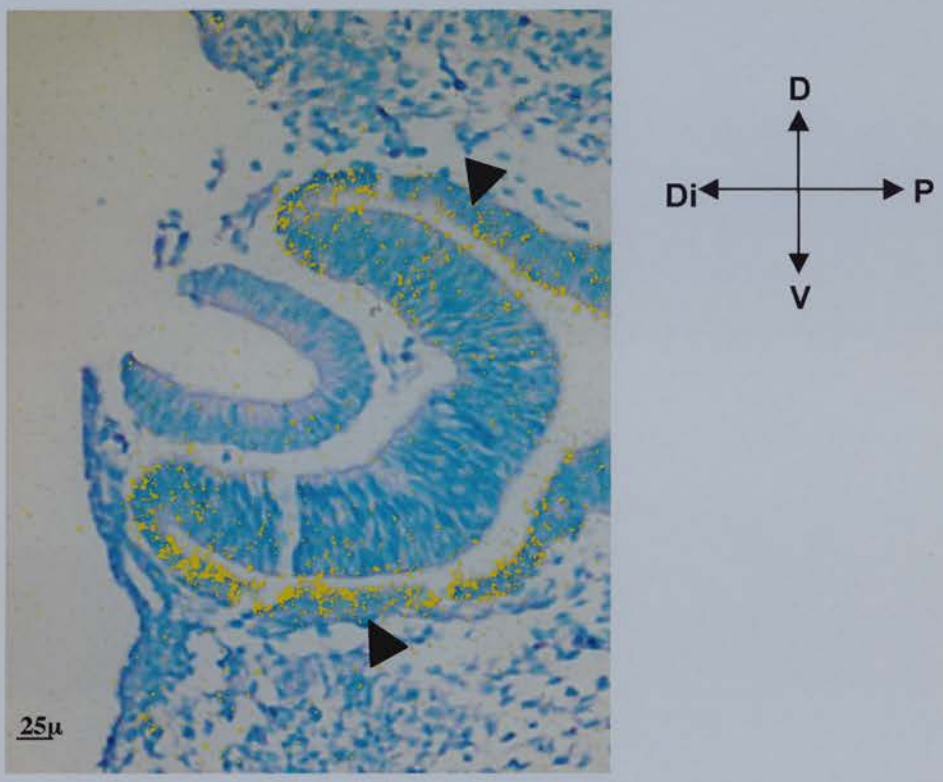


Fig 4.33 *Mitf* expression in eye region of the E10.5 transient transgenic mouse 4.2 which PCR analysis showed were carrying the transgene. Transverse section through the posterior half of the eye. *Mitf* expression was observed in the pigmented retinal epithelium, (arrowheads). Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

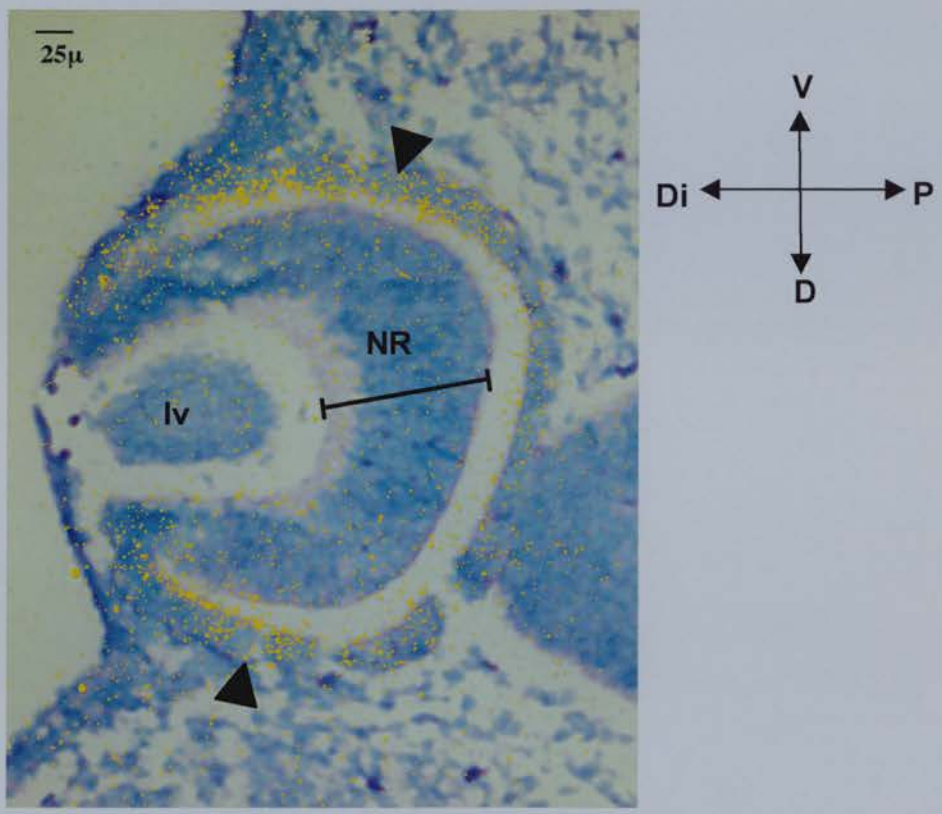


Fig 4.34 *Mitf* expression in eye region of a wildtype littermate of the E10.5 transient transgenic mice. Transverse section through the posterior half of the eye. *Mitf* expression was observed in the pigmented retinal epithelium, (arrowheads). NR= neural retina, lv = lens vesicle. Orientation; V=Ventral, D=Dorsal, P=Proximal, Di=Distal.

4.5 Summary and conclusions

There are several explanations for the small patches of βGal-positive cells observed in the PRE of one out of four p*Trp2*-driven transgenic lines (A81). The transgene may have been active in the majority of cells but poor translation from the IRES resulting in expression of the reporter gene in only a few cells. No expression of *Msx2* could be detected in the A81 embryos by *in situ* hybridization suggesting that the transgene was not active. The transgene may be silenced in most of the PRE cells in this line and in all the PRE cells in other lines as a result of autorepression. However, *Trp2* expression in the A81 embryos is normal suggesting that this was not the case. Aside from the general transgene silencing mechanisms which affect multiple copies of the transgene or are a consequence of integration into transcriptionally silent DNA, the p*Trp2* transgene may have been subject to silencing as a result of the IRESβGeo sequences or germline transmission.

Transgenic lines and transient transgenic embryos were generated with a p*Trp2Msx2* transgene from which the potential silencing influence of the IRESβGeo sequences had been removed. There was no evidence for transgene activity in the transgenic lines A204 and A205, suggesting that the lack of transgene activity is not a result of the IRESβGeo sequences. In 3 transient p*Trp2Msx2* transgenic embryos there was also no evidence for transgene activity or biological effects. This suggests that germline transmission is not silencing the transgene. In the future, following *in vitro* functional tests of the transgene, it will be important to confirm this by generating and investigating a larger number of transient transgenic embryos and stable transgenic lines.

Chapter 5 Discussion

The expression pattern of *Msx1* and *Msx2* during mouse eye development and the abnormal eye phenotype observed in the *Msx1*/*Msx2* double null mouse mutants suggests that these two genes have essential and yet redundant functions during mouse eye development. The Msx proteins are transcription factors with the potential to regulate genes both directly and via interactions with other proteins. The key to understanding the cellular functions of Msx1 and Msx2 during eye development is identifying the genes they regulate. In various different developmental contexts *Msx1* and *Msx2* have been associated with the regulation of cellular differentiation, proliferation, apoptosis and signalling pathways. Using a cell culture assay for Msx cellular function I have explored the formation of cells with a neural phenotype, the downregulation of Mitf and the effect on cell proliferation by *Msx1* and *Msx2 in vitro*. Here I discuss how this may help us to understand the cellular functions of Msx1 and Msx2 during vertebrate eye development.

During mouse eye development *Msx1* is expressed initially only in the perioptic mesenchyme surrounding the eye and, after eye cup formation, in the presumptive ciliary region at the distal tip of the neural retina (NR). In contrast, *Msx2* is expressed initially in the distal optic vesicle, the prospective neural retina and the prospective lens placode region of the surface ectoderm. Expression of *Msx2* continues in the prospective neural retina following optic cup formation but rapidly becomes restricted to the distal tip of the neural retina, where *Msx1* and *Msx2* are co-expressed. The expression of *Msx2* in the prospective NR domain of the optic vesicle suggests it has a role either in patterning NR or suppressing PRE cell fate. In chick PRE cells in culture, expression of mouse *Msx2* promotes the downregulation of the key pigmentation bHLH transcription factor Mitf and promotes the formation of cells with a neural-like phenotype. Under normal *in vitro* culture conditions untransfected PRE cells begin to dedifferentiate and display some characteristics of neural cells; a proportion of primary PRE cells in culture express the neural marker *TuJ1* and a few cells with a neural-like phenotype have been observed in control-transfected dedifferentiated PRE cultures. This suggests that PRE cells have predisposition to

develop neural characteristics. Forced expression of *Msx2* may be promoting PRE cell dedifferentiation to a multipotent cell state from which a few cells begin to differentiate down a neural pathway. The direct or indirect downregulation of *Mitf* by Msx2 could be enhancing the PRE dedifferentiation process.

During mouse eye development *Mitf* is expressed initially in the neuroepithelium of the entire optic vesicle but then becomes downregulated in the prospective NR domain and continues to be expressed in the prospective PRE (Bora *et al.*, 1998; Mochii *et al.*, 1998). In the mouse *Mitf*-null mutant the PRE expresses characteristics of the NR and fully differentiates into NR on the dorsal side (Kobayashi *et al.*, 1994; Nakayama *et al.*, 1998; Nguyen and Arnheiter, 2000). If PRE cells *in vivo* lack Mitf protein they develop NR characteristics. A requirement for mouse neuroepithelium cells to develop as NR cells may be downregulation of *Mitf*. A key question is does Msx2 have a role in the pathway downregulating *Mitf* in the prospective NR domain? In the mouse *Mitf* expression is seen at E9.0 throughout the neuroepithelium of the optic vesicle (Bora *et al.*, 1998; Nguyen and Arnheiter, 2000), *Msx2* expression at E9.0 has not been examined. At E9.5 *Msx2* is expressed in the distal optic vesicle (Monaghan *et al.*, 1991) where *Mitf* expression is no longer observed (Bora *et al.*, 1998; Nguyen and Arnheiter, 2000). Thus, the effects of ectopic expression of *Msx2* in PRE cells in culture and the expression pattern of *Msx2* and *Mitf* during development of the mouse eye fit with the hypothesis that a function of Msx2 in the prospective NR domain is the downregulation of *Mitf*. A priority was to confirm *in vivo* the effects of ectopic *Msx2* expression in culture. To investigate the *in vivo* effects of ectopic *Msx2* expression in PRE transgenic mice were generated. Once the technical problems of transgene expression discussed in Chapter 4 have been overcome these ideas could be investigated *in vivo* in a transgenic mouse model.

I have shown that expression of *Msx1* in PRE cells *in vitro* also promotes a decrease in Mitf expression and an increase in the formation of cells with a neural-like phenotype. This suggests that Msx1 and Msx2 may have similar cellular functions. Both Msx1 and Msx2 proteins may have similar molecular functions since the highly conserved homeobox mediates many of the interactions made by the proteins.

Furthermore, in progenitor cells in culture expression of both *Msx1* and *Msx2* had the same cellular effect of repressing differentiation by upregulating *cyclin D1* (Hu *et al.*, 2001). Evidence that Msx1 and Msx2 are functionally redundant during eye development comes from the reported phenotype of the $Msx1^{-/-}/Msx2^{-/-}$ double null mice (Rauchman *et al.*, 1997). An eye phenotype was reported in 100% of cases but is poorly characterized and has not been peer reviewed. The eye abnormalities range from no eyes to micropthalmia with a small lens vesicle and abnormal migration of mesenchyme into the optic cup. During the early stages of eye development *Msx1* is not co-expressed with *Msx2* raising the question of how Msx1 can compensate for the function of Msx2 in the $Msx2^{-/-}$ mouse mutants? One possibility is that Msx2 represses *Msx1* expression in the NR domain. In the absence of Msx2 in the $Msx2^{-/-}$ mutants expression of *Msx1* could be upregulated ectopically in the neural retina. Alternatively, Msx1 and Msx2 may functionally substitute for each other by acting at a distance. The perioptic mesenchyme is the closest tissue to the domain of *Msx2* expression in the prospective mouse NR that expresses *Msx1*. In rodents, mesenchymal cells are often observed between surface ectoderm and the distal part of the optic vesicle prior to lens placode induction (Bora *et al.*, 1998; de Iongh and Mcavoy, 1993; Furuta and Hogan, 1998; Kaufman, 1979). An Msx1-dependent signal from the perioptic mesenchyme could, therefore, have the same effects on the NR as intrinsic expression of *Msx2*. In *Msx2*-null mice the Msx1-promoted signal from the mesenchyme may thus be able to compensate for the absence of Msx2. Which signalling pathways could Msx1 and Msx2 be involved in? BMP4 is one candidate signal which could be regulated by Msx2 in the prospective NR and by Msx1 in the adjacent mesenchyme.

In contrast to the mouse, *Mitf* expression in the chick is seen only in the proximal cells of the optic vesicle, the prospective PRE (Nakayama *et al.*, 1998).
The expression pattern of *Mitf* in the chick raises the question, why is expression absent from the prospective NR domain? In contrast to the mouse, in the chick the distal part of the outgrowing optic vesicle is always in close contact with the overlying surface ectoderm (Hilfer, 1983). In the chick, *Msx2* is expressed in the surface ectoderm, but no expression of *Msx1* or *Msx2* is observed in the optic

neuroepithelium (Holme, 1998). A different mechanism must restrict *Mitf* expression in the chick. *Mitf* expression in the distal optic vesicle may be repressed by the diffusion of signalling molecules or direct cell interactions with the adjacent *Msx2*-expressing surface ectoderm. During development Msx1 and Msx2 may have functions both locally in the tissues where they are expressed and at a distance in adjacent tissues.

In the literature survey I have described the roles of Msx proteins in growth factor pathways of BMPs and FGFs during tooth, hindbrain and limb development. An essential function of Msx1 during tooth development is the regulation of BMP4 in dental mesenchyme, (Bei *et al.*, 2000) potentially by the formation of a positive feedback loop with BMP4 to signal to the dental epithelium. Since Msx proteins are believed to act as repressors, one way Msx1 could form a positive feedback loop with BMP4 in the dental mesenchyme is by repression of a BMP4-inhibitor, for example chordin or noggin. Soluble inhibitory proteins, which prevent receptor activation by binding ligand, have been found to be important for regulating the action of BMP-signalling (Massague and Chen, 2000; Smith, 1999). In the prospective NR, Msx2 may regulate BMP4 in a positive feedback loop and BMP4 may promote *Mitf* downregulation. Msx1 in the perioptic mesenchyme could also form a positive feedback loop with BMP4 by repression of a BMP4-inhibitor (See Fig 5.1 overleaf) In the *Msx2*-null mutant mice, BMP4 from the Msx1 positive feedback loop in the perioptic mesenchyme could signal to the adjacent optic vesicle neuroepithelium cells. Thus in the absence of Msx2, BMP4 from the adjacent mesenchyme could promote *Mitf* downregulation in the prospective NR cells. To investigate this, it would be interesting to examine BMP4 expression in *Msx*-transfected and control-transfected PRE cells and the effect of BMP4 on Mitf expression.

The regulation of BMP4 by Msx1 and Msx2 in the surface ectoderm may be essential for the development of the lens placode. In the preplacodal surface ectoderm BMP4 appears to be essential for the regulation of *Sox2* (Furuta and Hogan, 1998).

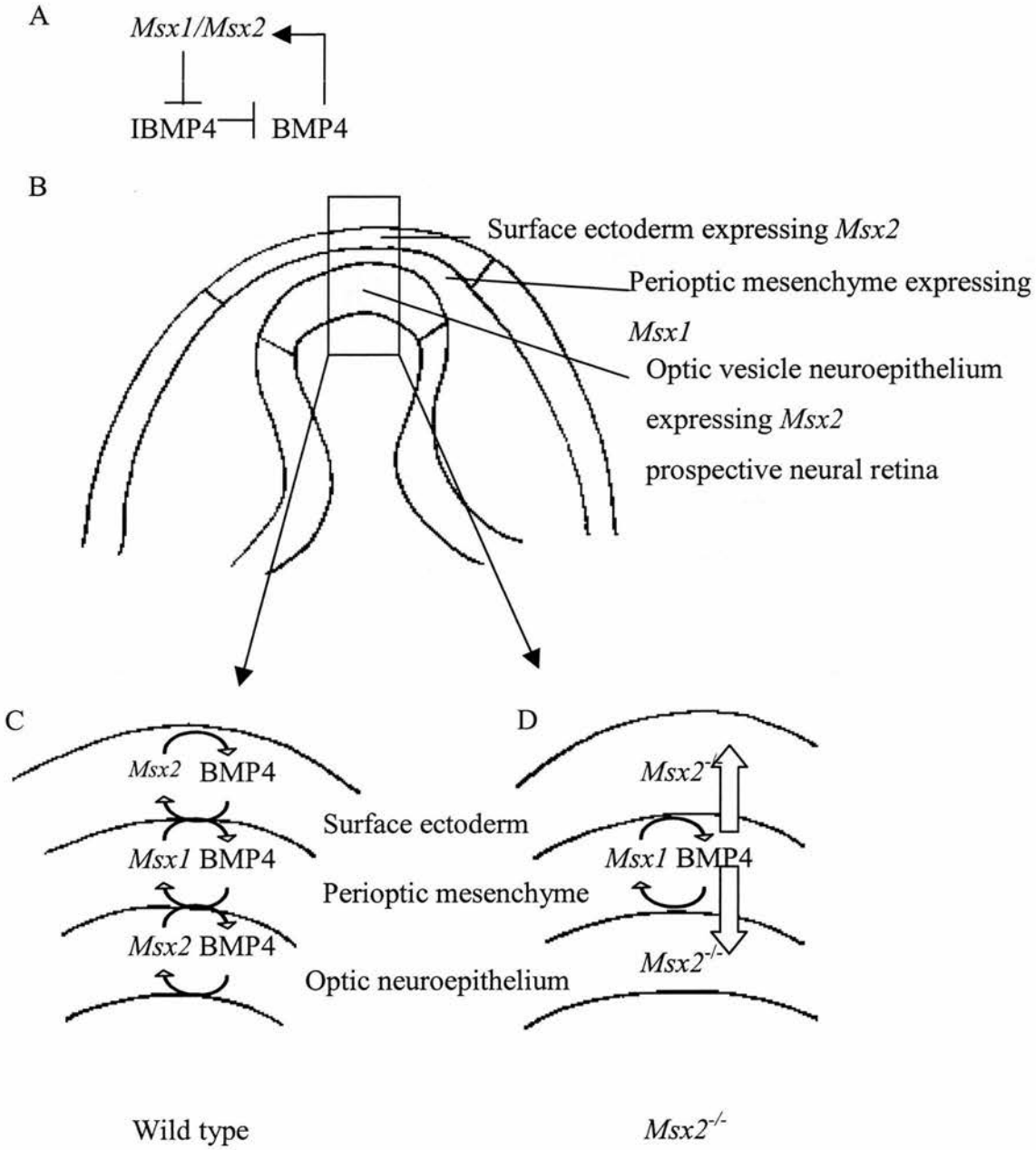


Fig 5.1 Schematic diagram summarizing a model for how *Msx1* and *Msx2* form positive feedback loops with BMP4 and how *Msx1* can compensate for *Msx2* in the surface ectoderm and optic neuroepithelium. A) proposed feedback loop between *Msx1/ Msx2* and BMP4 via repression of an inhibitor of BMP4 (IBMP4). B) Schematic diagram of tissues adjacent to optic vesicle neuroepithelium. C) *Msx2*/BMP4 positive feedback loops in surface ectoderm and distal optic vesicle (prospective neural retina) and *Msx1*/BMP4 positive feedback loop in perioptic mesenchyme. D) BMP4 from *Msx1*/BMP4 positive feedback loop in perioptic mesenchyme can produce BMP4 signal to both surface ectoderm and distal optic neuroepithelium.

Sox2 is in turn required for the regulation of *crystallin* genes in the lens placode (Kamachi *et al.*, 1995). Activation of *Sox2* in the preplacodal surface ectoderm may require a threshold level of BMP4-signalling. Msx2 may form a positive feedback loop with BMP4 in the prospective lens placode region by repression of a BMP4-inhibitor, resulting in BMP4-signalling in the preplacodal ectoderm reaching the threshold level for *Sox2* activation and lens development. In a similar manner to that described previously, in the perioptic mesenchyme Msx1 could form a positive feedback loop with BMP4 and supply BMP4 signal to the surface ectoderm. In the absence of Msx2, BMP4 from the mesenchyme could compensate and ensure the critical BMP4 threshold level is maintained. According to this speculative model, when both Msx1 and Msx2 are absent BMP4-signalling in the preplacodal surface ectoderm does not reach the critical threshold level for lens development. It would be interesting to test this model by analysis of BMP4 and the BMP4-inhibitors noggin and chordin expression in the heads of *Msx1*- and *Msx2*-double null mice. If BMP4 expression shows a reduction in these mice it would be interesting to attempt to rescue optic cup development by addition of BMP4 to cultured *Msx1*- and *Msx2*-double null optic vesicles.

Msx1 and Msx2 may have multiple functions in the prospective ciliary margin of the eye. Msx1 and Msx2 may be involved in maintaining the cells in a dedifferentiated state, by downregulating *Mitf*. The question also arises whether this role may involve regulation of cell proliferation, generating cells that contribute to the retina. Various lines of evidence from *in vivo* and cell culture suggest Msx1 and Msx2 stall cellular differentiation. Expression of *Msx1* in migrating dermal and muscle progenitors has been linked to maintaining their dedifferentiated state (Bendall *et al.*, 1999; Houzelstein *et al.*, 1999). During skull development and limb and digit regeneration Msx1 and Msx2 have been implicated in maintaining dedifferentiation and potentially facilitating proliferation (Endo *et al.*, 2000; Poss *et al.*, 2000; Reginelli *et al.*, 1995; Satokata *et al.*, 2000; Satokata and Maas, 1994). Recently, cell culture experiments have shown that Msx1 and Msx2 prevent progenitor cells from exiting the cell cycle by upregulating *cyclin D1* (Hu *et al.*, 2001). The prospective ciliary margin is the zone between the PRE and NR. *Msx1* and *Msx2* are expressed in the

ciliary margin when the rest of the optic cup starts to differentiate and may be involved in stalling the differentiation of these cells. The stalling of differentiation by Msx1 and Msx2 may be linked to the potential promotion of apoptosis in some cells of the ciliary margin of the chick (Trousse *et al.*, 2001). In addition, a small population of proliferating multipotent retinal progenitors have been discovered at the chick retinal margin (Fischer and Reh 2000). The expression of *Msx1* and *Msx2* may maintain some cells in the chick retinal margin in an actively proliferating state. In the *in vitro* assay I found no evidence of a direct role for Msx2 in promoting PRE cell proliferation. However, Msx1 and Msx2 appear to have an indirect role in the regulation of cell proliferation, preventing cells from exiting the cell cycle but not actively promoting proliferation. The cellular functions of Msx1 and Msx2 depend on the cellular context where they are expressed. In contrast to their roles in skull bone differentiation and limb regeneration, during limb and hindbrain development *Msx1* and *Msx2* have been linked to apoptosis (Chen *et al.*, 1997a; Ferrari *et al.*, 1998; Graham *et al.*, 1993; Takahashi *et al.*, 1998). Msx1 and Msx2 may trigger apoptosis by blocking essential cell survival signal pathways. This raises interesting questions about which pathways Msx1 and Msx2 are involved in and the molecular interactions they make in these pathways.

There is *in vitro* and *in vivo* evidence from studies in other systems that Msx1 regulates expression of the key transcription factor *MyoD,* potentially by direct DNA-binding or specific interference with homeodomain transcription factors which activate *MyoD* (Bendall *et al.*, 1999; Woloshin *et al.*, 1995). *MyoD*, in common with *Mitf*, is a bHLH transcription factor and their regulation by Msx1 may be mediated by similar interactions. *In vitro* studies suggest that interactions with both proteins and DNA are mediated by the homeodomain, which is conserved between Msx1 and Msx2 (Bendall *et al.*, 1998b; Bendall *et al.*, 1999; Newberry *et al.*, 1997; Zhang *et al.*, 1996; Zhang *et al.*, 1997). If Msx1 and Msx2 *in vitro* interactions represent those *in vivo* then the two proteins may make similar molecular interactions and therefore have similar cellular functions in some cells.

The results in cell culture and *in vivo* expression data suggest a cellular function of Msx2 in the prospective NR of the mouse may be the downregulation of *Mitf*. In this cellular function Msx1 may be functionally redundant with Msx2. From data and experiments in other systems a function of both Msx1 and Msx2 in the perioptic mesenchyme and optic cup neuroepithelium may be the regulation of BMP4 in positive feedback loops. The cell culture assay and transgenic mice provide approaches where the questions raised by this work about Msx pathways and candidate genes can be investigated. Does the regulation of *Mitf* represent an *in vivo* function of Msx2? Do Msx1 and Msx2 regulate *Mitf* by direct binding to it regulatory region? Do Msx1 and Msx2 downregulate *Mitf* by dimerization with homeodomain transcription factors which activate *Mitf*? Can BMP4 downregulate *Mitf*? Is BMP4 regulated by Msx1 and Msx2 in chick PRE cells in culture and is this via repression of a BMP4-inhibitor? What are the direct and in direct regulatory targets of Msx1 and Msx2 *in vitro* and *in vivo*?

5.1 Suggested direction of future work

The work described in this thesis raises interesting questions about the functions of Msx1 and Msx2 during vertebrate eye development. Firstly, does the downregulation of *Mitf* represents a cellular function of Msx2 *in vivo*? To investigate this an essential avenue to pursue in future work is the generation of an *in vivo* model of the *in vitro* assay. An alternative to the transgenic approach we took would be to clone *Msx2* and *Msx1* into high-titre retroviral expression vectors. The retrovirus could be used to infect the prospective PRE cells by injection into chick optic vesicles *in ovo* or mouse optic vesicles *in utero* or in culture, however this may be a technically challenging approach (Gaiano *et al.*, 1999; Nguyen and Arnheiter, 2000). An advantage of this method over transgenic mice is that ectopic expression of *Msx1* and *Msx2* is not dependent on the activity of a stably inserted transgene. However, it has the disadvantage that it is difficult to control the number and type of cells infected by the retrovirus. The *Trp2*-driven transgene ensures specific transgene expression in the PRE cells but before it is used to generate further transgenic mice it must be tested in cells in culture.

Of primary importance is to investigate in culture is whether Msx2 can to repress expression of *Trp2*. This could be addressed by transfecting PRE cells in culture with CMV*Msx2* or control construct and investigating expression of *Trp2* by RT-PCR or Trp2 by immunohistochemistry. These experiments could then be repeated using the p*Trp2Msx2* construct, to establish whether Msx2 was interfering with expression from the p*Trp2Msx2* transgene. Secondly, to test that the transgene is efficiently producing *Msx2,* p*Trp2Msx2*-transfected chick PRE cells can be examined for *Msx2* expression by RT-PCR or immunohistochemistry. If these experiments show that the p*Trp2Msx2* transgene actively produces *Msx2* transcript and protein *in vitro*, the transgene could be injected to generate, say ten, transgenic mouse lines. Transient transgenic embryos could be produced, as well as stable lines, to confirm whether germline transmission has an effect on transgene expression. The disadvantages of this approach are that several transgenic lines have to be produced to obtain a line with an active transgene and *in situ* hybridization has to be used to confirm ectopic *Msx2* expression in the PRE. An alternative to random transgene integration would be to introduce the *Msx2* gene into the mouse *Trp2* locus by homologous recombination in embryonic stem (ES) cells. The ES gene targetting approach has the important advantage that it selects for embryos and therefore lines with transgene activity. Once a transgenic line actively expressing *Msx2* in the PRE is obtained by either method any eye abnormalities would be characterized and the PRE investigated for a downregulation in *Mitf* expression. Both transgenic approaches could be used to introduce mutated versions of *Msx1* and *Msx2* to investigate how they effect their *in vivo* cellular functions.

The *Msx* mutations associated with human disease affect the function of Msx1 or Msx2 and it would be interesting to investigate these *in vivo.* The Pro148His mutation is associated with Boston-Type Craniosynostosis and lies is in the N-terminal arm of MSX1 a region associated with interactions with DNA, other transcription factors/DNA binding proteins and transcription machinery. The Arg31Pro mutation has been linked with selective tooth agenesis but the region of the homeodomain where it lies has not been associated with any molecular

interactions. These mutations could be introduced into the *Msx2* homeobox by PCR and the transgenic mice generated either by random transgene insertion or gene targetting in ES cells. The effects of these mutations on the *in vivo* cellular functions of Msx1 and Msx2 in the optic neuroepithelium of the transgenic mice could be investigated by analysing *Mitf* expression by *in situ* hybridization. An alternative to the production of transgenic mice, for investigating the *in vivo* functions of Msx1 and Msx2 during eye development, is to examine the *Msx1/Msx2* single and double null mutants.

In the discussion I have presented a model where during early eye development Msx1 and Msx2 regulate *Mitf* and BMP-inhibitors in the surface ectoderm, perioptic mesenchyme and optic neuroepithelium. The expression of *Mitf*, BMP4, chordin and noggin could be investigated by *in situ* hybridization in the *Msx1/Msx2* single and double null mice to provide evidence for the relationships between Msx1, Msx2 and *Mitf* proposed in this model. In parallel with these *in vivo* experiments the specificity of *Mitf* regulation by Msx1 and Msx2 can be explored in the physiologically relevant cell culture assay.

One of the interesting results I found with primary chick PRE cells is that both Msx1 and Msx2 downregulate *Mitf* and promote formation of dendritic cells. This raises the question; are the cellular effects of *Mitf* downregulation and dendritic cell formation specific to Msx1 and Msx2? The specificity of these cellular effects could be investigated by expressing the engrailed protein in PRE cells from a CMV-driven expression vector. In addition, it would be interesting to produce *Msx1* and *Msx2* expression constructs containing the Pro148His and Arg31Pro mutations, which are known to affect MSX function in humans. Transfection of these may affect the downregulation of Mitf and formation of dendritic cells in the cellular assay. Further *in vitro* experiments would be required to establish how the Pro148His and Arg31Pro mutations affects interactions with target DNA, transcription factors and transcription machinery and therefore cellular function. Providing the cellular effects seen in the assay are specific to the Msx proteins how can these be used to further investigate Msx downstream genes?

Interesting questions are raised in this thesis about how Msx1 and Msx2 regulate *Mitf,* is it direct or indirect and what other genes do they regulate? These questions could be investigated by the application of molecular techniques to the cell culture assay. DIG *in situ* on *Msx2*-transfected PRE cell in culture could be used to identify Msx2 downstream genes. The disadvantages of this approach are that it is limited to candidate genes and those for which chick *in situ* probes are available, furthermore, this method may only be able to detect relatively large changes in gene expression. When Msx1 and Msx2 promote the neural phenotype in PRE cells they may be doing so via known or unknown genes. *Mitf* may be one of several regulatory targets of Msx2 in the optic neuroepithelium. How could Msx1 and Msx2 form positive feedback loops with BMP4 during development of the tooth and eye? Experiments with progenitor cell lines have shown that Msx1 and Msx2 regulate *cyclin D1* indirectly, which prompts the question what are the direct regulatory targets of Msx1 and Msx2? The key to understanding how Msx1 and Msx2 regulate cellular differentiation, division and apoptosis is to determine the genes whose expression they regulate both directly and indirectly.

Msx1 and Msx2 downstream genes could be identified by applying molecular differential screening approaches (for example, RDA or DD-RT-PCR) to mRNA extracted from the transfected cells. A limitation of using primary cells or cells derived from primary cells, for further investigation of the cellular functions of Msx1 and Msx2, is the relatively small number of transfected cells and small amount of material which can be extracted from them. The number of cells in the assay could be substantially increased by development of an inducible *Msx*-expressing stable cell line. mRNA extracted from cells before and after induction could be compared to identify direct, indirect, known and unknown downstream targets. However, as a result of the transformation the cellular characteristics of a cell from a cell line may be significantly altered, so that it no longer accurately represents the *in vivo* cellular context of the protein of interest. This was confirmed by some preliminary experiments I undertook to investigate Mitf downregulation by Msx2 in the human APRE19 cell line. The cells from the stable PRE cell line showed a different pattern

of Mitf expression to chick primary PRE cells showing that cells from a cell line may not accurately represent the cellular context where Msx2 and Msx1 are active. Despite being limited by the number of cells the results obtained with primary cells reflect the *in vivo* cellular context of Msx1 and Msx2. Microarray technology, which utilizes very small quantities of mRNA, has the potential to be applied to material extracted from primary cells.

The first step in using microarrays to investigate Msx downstream genes is to separate *Msx1*- or *Msx2*-transfected cells. Green fluorscent protein (GFP) could be cloned into the *Msx1*, *Msx2* and control expression constructs allowing Fluorescence Activated Cell Sorting of transfected cells, (Ormerod, 2000). The mRNA from the control- and *Msx*-transfected cells could be extracted, amplified, fluorscently-labelled and used to probe a microarray chip of all genes normally expressed in chick PRE cells. Direct and indirect Msx downstream genes would be repressed in the *Msx*-transfected cells when compared to control-transfected cells. Furthermore, the overall gene expression profile from the *Msx*-transfected cells when compared to control-transfected cells may reveal whether the Msx proteins are activators or repressors, since it would give a global picture of the effect of Msx on gene expression. However, it may be difficult to draw such a simple conclusion from the results of these experiments, because some of the target genes of Msx proteins may code for gene expression repressors and so the net effect would be upregulation. An adaptation of this approach, using a modified *Msx* construct, could be used to investigate the question raised by the results in cell culture; do both Msx1 and Msx2 regulate *Mitf* directly and to identify direct regulatory targets of these genes.

A construct could be produced expressing the Msx homeodomain fused to the VP16 activation domain (Friedman *et al.*, 1988). If the Msx homeodomain binds directly to *Mitf* its expression would be expected to be upregulated by the *Msx*VP16 construct when compared with a control construct. In a complementary experiment the Msx homeodomain could be fused to repressor domain of Engrailed (En^r) (Badiani *et al.*, 1994; Bao *et al.*, 1999; Conlon *et al.*, 1996; Furukawa *et al.*, 1997; Yu *et al.*, 2001). *Msx* En^r would be expected to repress *Mitf* when compared with control constructs if

Mitf is bound directly by the Msx homeodomain. Chick PRE cells transfected with *Msx*VP16 and *Msx* En^r and control constructs could be examined for *Mitf* or Mitf expression by *in situ* or immunohistochemistry. The advantages of these experiments over *in vitro* gel shift assays are that they use the physiologically relevant PRE cell context, in which Msx1 and Msx2 are known to have an effect. However, when interpreting the results of these experiments it may be difficult to distinguish direct action of Msx1 and Msx2 on *Mitf,* from indirect action via a *Mitf* repressor and/or activator, illustrated in diagram below.

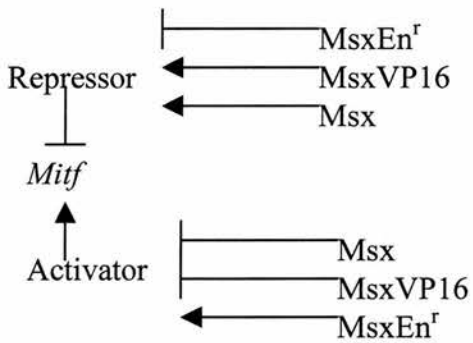


Fig 5.2. The possible indirect actions of Msx on Mitf via a repressor and/or activator. The effects *Msx*VP16 and *Msx* En^r could have.

If Msx1 or Msx2 activates a repressor of *Mitf* expression and/or represses an activator of *Mitf* expression by direct binding, they may repress Mitf without a direct interaction with *Mitf.* It may not be possible to distinguish between this and direct binding with the *Mitf Msx*VP16 and *Msx* En^r constructs. As shown in Fig 5.2, *Msx*VP16 would activate the *Mitf* repressor and activator and/or *Msx* En^r would repress the *Mitf* repressor and activator. This would result in repression of *Mitf* without a direct interaction between Msx and *Mitf.* Furthermore, activation of inhibitor may give inhibition even if activator is activated. In addition, inhibition of activator may also result in some activation if inhibitor is also inhibited. The net effect on *Mitf* would depend on the balance of actual interactions, but may still produce *Mitf* upregulation or downregulation without direct interaction with *Mitf.* One way direct interaction between Msx1 or Msx2 and *Mitf* could be tested would be

by fusing Mitf's regulatory region to a reporter gene could be co-transfected with *Msx1* and *Msx2* *Msx*VP16GFP and *Msx* En^r GFP. Any apparent direct interaction could be tested in vivo by introduing mutations in the binding sites on the Msx protein and *Mitf* regulatory region. It may be possible to identify unknown direct regulatory targets of the Msx proteins by combining these *Msx*VP16 and *Msx* En^r constructs with GFP, FACS cell sorting and microarray technology.

GFP could be included in the *Msx*VP16 and *Msx* En^r constructs which could be sorted by FACS, their RNA extracted and amplified, labelled and used to probe the PRE microarray. Direct Msx candidate genes may be upregulated in *Msx*VP16GFP cells and downregulated in *Msx* En^r GFP-expressing cells. Genes showing downregulation in *Msx*-transfected cells but not regulated in *Msx*VP16GFP or *Msx* En^r GFP could represent indirect Msx regulatory targets. The advantage of this combined approach is that it may allow identification of direct and indirect targets of Msx proteins. However, it should be noted that these methods will not help identify genes which are regulated by Msx binding DNA directly or in a complex bound to the DNA. These could be identified by applying these approaches to the Msx DNA-binding proteins and transcription factors and *in vivo* confirmation of interactions. The expression of Msx candidate downstream genes identified by microarray could be investigated *in vivo* by *in situ* hybridization on *Msx1* and *Msx2* single and double knockout mice tissue. The molecular interactions of Msx1 and Msx2 on direct Msx candidate downstream targets identified on the microarray could be investigated in the *in vitro* assay.

The *in vitro* culture assay explored in this thesis provides a means to investigate the cellular functions and downstream regulatory targets of Msx1 and Msx2. Further work with this system *in vitro* and *vivo* may reveal both direct and indirect downstream genes. These results, coupled with the growing understanding of the complex network of interactions that regulate vertebrate eye development, could help understand the roles Msx1 and Msx2 have in this and other developmental processes.

Appendix 1

Construct maps

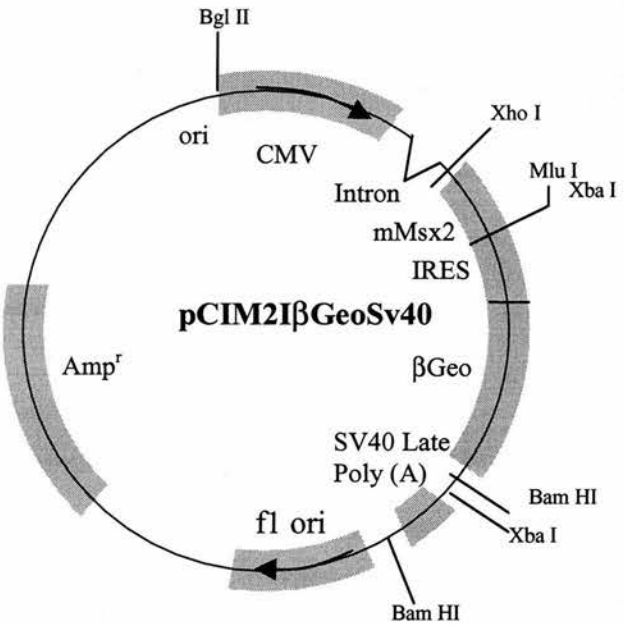


Fig A1.1. CMV m*Msx2* expression construct for transfection of cells in culture

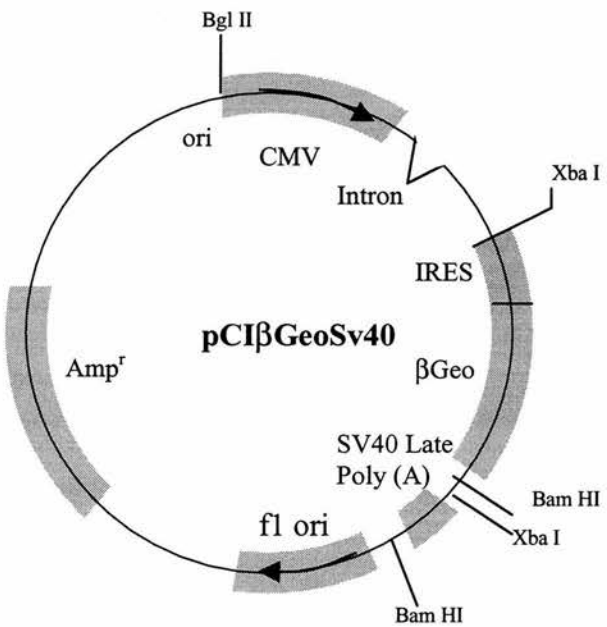


Fig A1.2. Control CMV construct without m*Msx2*

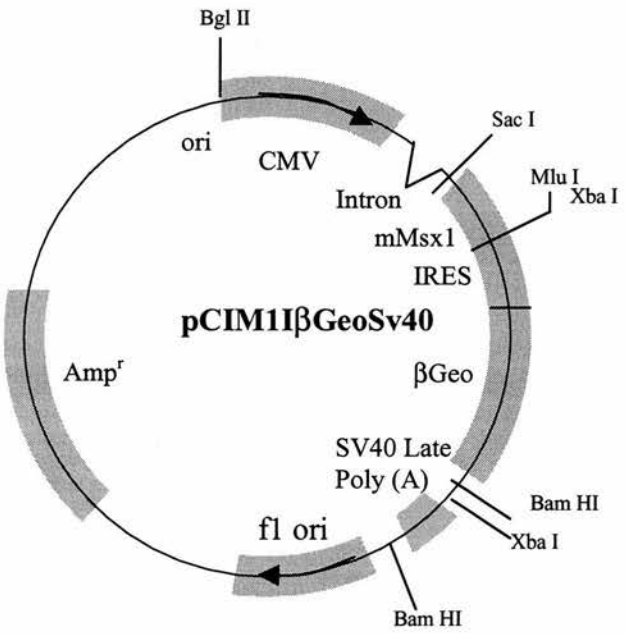


Fig A1.3. CMV m*Msx1* expression construct for transfection of cells in culture

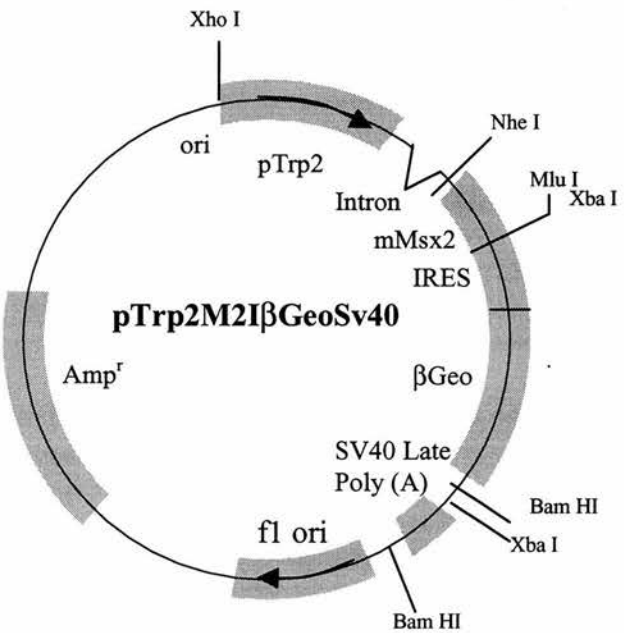


Fig A1.4. p*Trp2* m*Msx2* expression construct for transfection of cells in culture and for production of transgenic mice

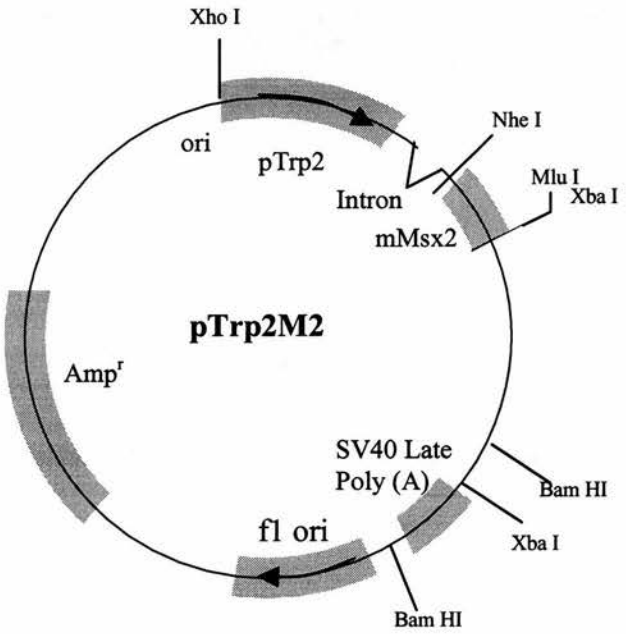


Fig A1.5. p*Trp2* expression construct without IRES/βGeo for production of transgenic mice.

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